TISSUE CULTURE AND MORPHOGENESIS OF *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red*

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ASBTRACT

In vitro regeneration of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red*, also known as Begonia Rose, an ornamental plant was achieved from four different intact explants; such as leaf, peduncle, petiole and stem explants. The sterilization protocol was established for *in vivo* explants to overcome contamination. The results revealed that four different types of explants could produce *in vitro* regeneration. Leaf and petiole explants were amongst the best explants that were observed in this study. The optimum regeneration medium was identified in this study, i.e. MS medium supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA which produced normal shoots.

Treatment of MS medium supplemented with lower concentration of BAP (0.1 mg/l) and NAA (0.1 mg/l) produced less normal shoots, whereas higher concentration of BAP (2.0 mg/l) and NAA (2.0 mg/l) produced micro shoots. No shoot could be obtained in the MS media devoid of hormone and also in MS supplemented with various concentrations of BAP applied singly. Root induction were obtained in the MS medium supplemented with various concentrations of NAA and MS media fortified with 0.5-1.0 mg/l NAA could produce optimum root induction. TDZ is known to have high cytokinin-like activity was identified as effective in the production of abnormal micro shoots, whereas combinations of different types of auxins and cytokinins could also produce *in vitro* shoots.

Optimization of callus induction was also established in Begonia. Two different types of explants were selected in this study i.e. leaf and petiole explants. Optimum callus could be obtained in MS supplemented with 1.0 mg/l BAP and 0.5 mg/l 2,4-D. Green and yellowish, compact nodular callus were obtained during callus induction. Treatment of

MS media fortified with different concentrations of 2,4-D (0.1-1.0 mg/l) produced yellowish nodular callus, whereas green nodular callus were obtained in MS media with combinations of BAP (1.0 mg/l) and 2,4-D (0.1-0.5 mg/l).

Direct somatic embryos was also induced from leaf and petiole explants of Begonia. The stock explants derived from *in vitro* plantlets that were subcultured into MS media supplemented with 1.0 mg/l TIBA. By using MS solid media supplemented with 1.0 mg/l BAP, 0.1 mg/l 2,4-D and 500 mg/l casien hydrolysate, green nodular callus were induced from the explants. The nodullar structures from leaf explants were further developed into heart-shaped, torpedo-shaped and cotyledonary-stage upon transferred to development media (MS media supplemented with 0.5 mg/l GA_3).

The optimum callus produced in Begonia was further cultured into suspension cultures to mass-produce the callus. The results showed that embryogenic callus could multiply in the MS liquid media supplemented with 1.0 mg/l BAP in combination with 0.1 mg/l 2, 4-D. A new protocol was identified to propagate callus for regeneration purposes. The suspension cultures containing cotyledonary-stage structures were subcultured onto MS solid media supplemented with 0.5 mg/l GA_3 for further development of the embryoids.

In this study, production of synthetic seeds was also attempted. Somatic embryos and micro shoots were encapsulated in 3.0% (w/v) of sodium alginate solution for the production of synthetic seeds. The synthetic seeds which were stored at 4 °C for 1-6 months were successfully germinated onto MS basal media. The germination rate was up to 83.33%. The synthetic seeds were also successfully germinated under *in vivo* conditions on topsoil 1 and vermiculite but not on the sphagnum.

In vitro flowering was also investigated in Begonia using three different explants i.e. inflorescences, peduncles and petals. The results obtained showed that *in vitro* flowering could be obtained from inflorescence explants cultured onto MS media supplemented with 1.0 mg/l BAP, 1.0 mg/l NAA, 40 mg/l adenine and 4.0% (w/v) sucrose. The abnormal red inflorescences obtained devoid of reproductive organs and did not develop into mature flowers upon transfer to MS media supplemented with 0.5 mg/l GA_3.

All regenerated plantlets obtained from several tissue culture techniques including *in vitro* regeneration, suspension cultures, somatic embryos, synthetic seeds and *in vitro* flowering were transferred to the greenhouse for further development. Four different types of substrates were used in this study. The results showed that topsoil 1, topsoil 2 and sphagnum could be used as substrates. Almost all regenerants produced flowers after 9 months being transplanted in the greenhouse. However, regenerants that being acclimatized in the tissue culture room (25 ±1ºC; 16 hours light and 8 hours dark) did not produce flowers even after 12 months of incubation period. SEM and some morphological studies proved that all regenerants derived from *in vitro* culture systems were morphologically similar with intact (parent) plant of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red*.

ABSTRAK

Regenerasi *in vitro* telah diperolehi bagi tumbuhan hiasan *Begonia* x *hiemalis* Fostch. kultivar *Schwabenland Red,* atau dikenali sebagai Begonia Ros, menggunakan empat jenis eksplan 'intact' yang berbeza iaitu daun, tangkai bunga, petiol dan juga batang tumbuhan *in vivo*. Protokol teknik pensterilan untuk mengatasi kontaminasi dari tumbuhan *in vivo* dapat dihasilkan. Keputusan yang diperolehi membuktikan bahawa keempat-empat eksplan yang berbeza memberikan respons ke atas regenerasi *in vitro* dan eksplan daun dan petiol merupakan eksplan yang terbaik yang dihasilkan dalam kajian ini. Media optima bagi regenerasi dikenalpasti dalam kajian ini, iaitu media MS dengan kombinasi hormon 1.0 mg/l BAP dan 1.0 mg/l NAA, di mana pucuk normal dapat dihasilkan.

Media MS dengan kepekatan hormon BAP (0.1 mg/l) dan NAA (0.1 mg/l) yang rendah menghasilkan kuantiti pucuk normal yang rendah manakala, konsentrasi BAP (2.0 mg/l) dan NAA (2.0 mg/l) yang tinggi menghasilkan pucuk mikro. Eksplan yang dikultur di dalam MS tanpa hormon ataupun dengan kepekatan BAP tidak menghasilkan pucuk. Induksi akar berjaya dihasilkan pada eksplan yang dikultur dalam media MS media yang mengandungi NAA. Media MS dengan 0.5-1.0 mg/l NAA merupakan media optima untuk penginduksian akar. Penggunaan hormon TDZ pula berjaya menginduksi pucuk mikro abnormal pada eksplan. Manakala kombinasi auksin dan sitokinin berbeza juga mampu menghasilkan pucuk *in vitro* pada semua eksplan.

Penginduksian kalus optima juga dikenalpasti dalam Begonia. Dua jenis eksplan digunakan dalan kajian ini iaitu eksplan daun dan petiol. Kalus optima diperolehi dalam kombinasi media MS dengan 1.0 mg/l BAP dan 0.1 mg/l 2,4-D. Kalus yang diperolehi

berwarna hijau dan kekuningan, padat dan bernodul. Media MS yang mengandungi hormon 2,4-D (0.1-1.0 mg/l) sahaja yang menghasilkan kalus kekuningan manakala kalus hijau yang bernodul dihasilkan dari media MS dengan kombinasi hormon BAP (1.0 mg/l) dan 2,4-D (0.1-0.5 mg/l).

Penginduksian embrio somatik secara langsung turut diperolehi dari daun dan petiol yang dikultur pada media MS yang mengandungi 1.0 mg/l BAP, 0.1 mg/l 2,4-D and 500 mg/l casien hydrolysate. Tumbuhan stok yang digunakan merupakan plantlet *in vitro* yang disubkultur di dalam media MS yang mengandungi 1.0 mg/l TIBA. Kalus bernodul hijau berjaya diinduksikan dari eksplan daun dan kalus nodul terus berkembang kepada struktur hati, torpedo dan kotiledon apabila dipindahkan kepada media perkembangan iaitu MS media yang mengandungi 0.5 mg/l GA_3.

Penghasilan kalus optima digunakan seterusnya untuk penghasilan kultur ampaian. Keputusan menunjukkan bahawa kalus embriogenik mengganda dalam media MS dengan kombinasi 1.0 mg/l BAP dan 0.1 mg/l 2,4-D. Kultur ampaian yang dihasilkan menghasilkan struktur embrio peringkat kotiledon dan disubkultur ke dalam media MS yang mengandungi 0.5 mg/l GA_3 untuk perkembangan seterusnya.

Dalam kajian ini biji benih tiruan juga telah berjaya dihasilkan. Embrio somatik dan pucuk mikro turut dikapsulkan di dalam 3.0% (w/v) larutan sodium alginate untuk menghasilkan biji benih tiruan. Biji benih tiruan yang dihasilkan disimpan pada suhu 4 °C selama 1-6 bulan. Keputusan menunjukkan bahawa biji benih yang disimpan selama 1-3 bulan sahaja yang mampu bercambah di atas media MS tanpa hormon dengan kadar percambahan

mencecah kepada 83.33%. Biji benih tiruan yang dihasilkan juga mampu bercambah secara *in vivo* menggunakan tanah kebun 1 dan vermiculite tetapi tidak di atas sphagnum.

Penginduksian pembungaan secara *in vitro* bagi Begonia juga dikaji dengan menggunakan tiga eksplan berbeza iaitu kudup, tangkai bunga dan juga petal. Keputusan yang diperolehi menunjukkan bahawa pembungaan *in vitro* berjaya dihasilkan daripada kudup bunga yang dikultur pada media MS dengan komposisi 1.0 mg/l BAP, 1.0 mg/l NAA, 40 mg/l adenine dan 4.0%(w/v) sukrosa. Kudup bunga abnormal berwana merah tanpa organ pembiakan berjaya dihasilkan dan ianya tidak berkembang kepada bunga matang setelah dipindahkan ke atas media MS yang mengandungi 0.5 mg/l GA_3.

Plantlet yang berjaya dihasilkan dari pelbagai cara menggunakan kultur tisu termasuklah regenerasi *in vitro* secara langsung, kultur ampaian, embriogenesis somatik, biji benih tiruan dan pembungaan *in vitro* berjaya dipindahkan ke tanah untuk tujuan aklimatisasi. Terdapat empat jenis tanah digunakan dalam kajian ini dan keputusan menunjukkan bahawa tanah kebun 1, tanah kebun 2 dan sphagnum amat sesuai sebagai substrat. Tumbuhan ini berjaya menginduksikan bunga selepas 9 bulan dipindahkan ke tanah. Walau bagaimanapun, regeneran yang diaklimatisasikan di dalam bilik kultur (25 ± 1ºC; 16 jam cahaya dan 8 jam gelap) tidak menghasilkan bunga selepas tempoh 12 bulan. Kajian SEM dan morphologi juga menunjukkan bahawa regeneran dari pelbagai sistem *in vitro* yang dihasilkan mempunyai ciri-ciri morfologi yang serupa dengan tumbuhan induk *Begonia* x *hiemalis* Fotsch. kultivar. *Schwabenland Red*.

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LIST OF ABBREVIATIONS

BAP	Benzyl amino purine
cv.	cultivar
2,4-D	2,4- Dichlorophenoxyacetic acid
ANOVA	Analysis of variance
CIM	Callus induction medium
EDM	Embryo development medium
FWC	Fresh weight of callus
GA_3	Giberellic acid
IAA	Indole-3-acetic acid
IBA	Indolebutyric acid
2-iP	2-isopentenylaminopurine
Kinetin	6-furfurylaminopurine
kPa	Kilo Pasca
mg/l	Milligram per liter
min	Minute
MS	Murashige and Skoog
NAA	Naphthalene acetic acids
NAA	Napththalene acetic acid
PCM	Plant culture medium
Rpm	Rotation per minute
SCM	Suspension culture medium
SEIM	Somatic embryo induction medium
SEM	Scanning electron microscope

TDZ	Thiadiazuron
TIBA	2,3,5-triioobenzoic acid
Tween 20	Polyoxyethylene sorbitan monolaurate
v/v	Volume per volume
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

1.1 GENERAL DESCRIPTION OF BEGONIA

Begonias, known as versatile temperate plants comprise an enormous group of lovely and very spectacular flowering plants. They are popular and marketed as flowerbeds, for hanging baskets, as pot plants, and for indoor houseplants. Begonias are prized equally for their dainty clusters of blossoms as well as their cheerful and interesting leaves. When they are not in bloom, which is infrequent once established, their attractive, waxy green or chocolate colored leaves show themselves off in the garden.

The primary center of diversity for the genus is the neotropics (Mexico to Argentina); a second center of diversity is in south central Asia (India, Sri Lanka, and China). Takhtajan (1981) found that the greater part of the Begonia family also can be found in North America. Some species are native to the southern, southeastern, and western parts of Africa. They are also native to South Asia and a distribution that extends from the temperate region up to Asia especially in the high lands. In Malaysian gardens, Begonias have been cultivated for many decades and also known as “Asam Batu” or “Resam Batu”.

1.1.1 Classification and Nomenclature of Begonia

Division:	Magnoliophyta / Angiospermae
Class:	Magnoliatae / Dicotyledones
Subclass:	Dilleniidae
Super order:	Dillenianae
Order:	Begoniales (Datiscales)
Family:	Begoniaceae
Genus:	Begonia
Species:	*Begonia* x *hiemalis* Fotsch.
Cultivar:	*Aphrodite Peach, Krefeld Orange, Schwabenland Pink, Schwabenland Red,*

Begonias belong to the family Begoniaceae which consists of 5 genera and 920 species (Hickey and King, 1981). The genus *Begonia* L. (family Begoniaceae) comprises more than 1 000 species, native to tropical regions of Africa, Central and South America and Asia, as well as a great number of horticultural hybrids and cultivars (Simmonds, 1992). There are about 5 000 varieties and cultivars that can grow all over the world. Simmonds (1992) has identified that Begonias of major commercial interest can be classified into five horticultural groups and each of these groups are cytologically diverse. They are known as fibrous rooted Begonia, rhizomatous Begonia, tuberous Begonia, Christmas Begonia and Elatior Begonia. The fibrous rooted begonia is used as bedding plants such as *Begonia semperflorens* Link and Otto (2n = 33-66). The rhizomatous begonia is used as foliage

plants such as *Begonia rex* Putz. (2n = 32-34). The third group known as tuberous begonia and these are flowering pot plants and garden specimens. *Begonia* x *tuberhybrida* Voss (2n = 27-56) is one of the example from this group. The next group known as Christmas Begonia used as a potted plant such as *Begonia* x *cheimantha* Everett (2n = 27-54). The last group and was selected in this study known as Elatior Begonia, *Begonia* x *hiemalis* Fotsch. (2n = 35-63) that normally used as potted flowering plants.

Begonia x *hiemalis* Fotsch. or Elatior Begonia is winter flowering Begonia and developed from crosses between *B. socotrana* Hook. and *B.* x *tuberhybrida*. The *Begonia* x *hiemalis* Fotsch. plant is normally with thick, shiny and dark green leaves. The dark green leaves are ornamental in shape. They are popularly known as Begonia Rose because of rose-like flower with shaded-tolerant pot plants. The flowers are large and mostly double and come in colors of orange, pink, red and yellow. The inflorescences consist of single and double flowers arranged in loose clusters that rise above the foliage. They are a fibrous-rooted Begonia, which can be used for cuttings.

1.1.2 Morphological Description of Begonia

Few plant families exhibit the diversity in size, growth habit, and foliage morphology that is found within the genus *Begonia*. Most of the Begonias are perennial, monoecious herbs, with succulent stems and thick rhizomes or tubers. The leaves are radical or alternate in 2 ranks, usually asymmetric, with large membranous stipules. Leaves range in size from under 2 cm to over 50 cm. The stems are fleshy and the leaves are all lop-sided. The leaf surfaces vary from glabrous to densely hairy. The inflorescence are usually axillaries with unisexual and zigomorphic flowers. Flowers are predominantly white, but may be pink, red, orange, or rarely yellow. Flowers are characteristically monoecious with separate male and female flowers, and the pistillate flowers have inferior ovaries. The perianth has 2 to many segments and known as petaloid. The stamens are numerous, ovary inferior, of usually 3 united carpels, 3-locular, with numerous anatropous ovules on axile placentas and styles distinct or basally connate. The fruit is papery, leathery, or fleshy and usually a loculicidal, winged capsule rarely a berry. The seeds are numerous and minute without endosperm (Hickey and King, 1981). The basic chromosome numbers (x) for the genus are 6, 7, and 13. Chromosome numbers range from 2n = 28 to 66. The vegetative characteristics of Begonias with a fibrous-rooted, half-hardy, more or less erect, succulent perennial often treated in garden as an annual, which reaches a height of 15 to 45 cm. The stem is glabrous, fleshy, reddish green and bears opposite leaves. The leaves are glabrous, roundish ovate, more or less oblique at the base and have serrulate, ciliate margins (Hickey and King, 1981).

1.1.3 Propagation and Planting of Begonia

Begonias are usually propagated from seeds or vegetative means i.e. stem cuttings and leaf cuttings. Seeds are very fine, dust-like, and take two to three weeks to germinate. Many people prefer to buy seedlings rather than try to germinate these tiny seeds. Simmonds (1992) has classified five different horticultural groups of Begonias and found that most fibrous rooted Begonias are propagated from seeds although additional plants can be obtained by taking stem cuttings from stock plants. Rhizomatous Begonias are most convenient propagated vegetatively by leaf vein cuttings and are grown for their foliage characteristics. The tuberous begonia can be propagated vegetatively by tubers especially for triploid hybrids although seed propagation is possible for the diploid hybrids. Propagation of Christmas Begonia is by leaf petiole and stem cuttings. Elatior Begonia is sterile and propagated by leaf petiole cuttings and terminal cuttings (Simmonds, 1992).

Cuttings are much easier for propagation of a few plants. Separating and replanting the tuberous roots commonly propagate tuberous varieties. Unfortunately, this type of Begonia is hard to maintain after they flower in the summer months. It is best to consider them a winter color display and discard the plant after the flowers have ceased. Simmonds (1992) has found the conventional methods of vegetative propagation are applied to all group of commercially important Begonias in order to maintain desirable hybrid traits.

1.1.4 The Economic Importance of Begonia

About 200 species have been introduced by commercial growers, and among them *Begonia* x *tuberhybrida*, *Begonia* x *hiemalis*, *Begonia* x *elatior*, *Begonia* x *cheimantha* and *Begonia* x *socotrana* are important species (Takayama, 1983). Holdgate (1977) reported that, Begonia ranked number 14 out of the total sales value in United States for florist crops, which give about $3 409 501 US. In 1993, Begonia ranked number four in the total percentage (7.5%) of crop mix with respect to seed-propagated plants used for bedding plant production. This translates into a gross wholesale value of approximately $49 million.

Other than great horticultural values, Begonias also has medicinal values. Phytochemical investigation of various extracts of the leaves of *Begonia malabarica* Lam. resulted in the isolation and identification of six known compounds, viz. friedelin, epi-friedelinol, β-sitosterol, luteolin, quercetin, β–sitosterol-3-β-D-glucopyranoside (Ramesh *et al.*, 2002) and stigmasterol. The leaf of *Begonia* x *semperflorens-cultorum* Hort. contained lutein and β–carotene whereas *Begonia* x *cheimantha* Everett yielded 30-300 μg kinetin equivalents per kg of fresh leaves. The leaves are also substituted for tamarind, the boiled leaves can be used for stomach ulcer, stomachache, diarrhea and respiratory problems (Ramesh *et al*., 2002). Based on studies on biological activities, some Begonias have the ability to inhibit process the antibacterial and antifungal activities (Ramesh *et al*., 2002).

1.1.5 The Limitation of Begonia

Most Begonia species also do not lend themselves to bedding plant production due to undesirable habit, a long cropping period, or poor seed set. *Begonia* x *hiemalis* Fotsch. also do not produce seeds. Most of these species, therefore, are neglected by hobbyist or botanical collections and are propagated vegetatively.

Although Begonias can be readily vegetatively propagated, they are susceptible to many pathogenic bacteria, fungi and nematodes (Castillo and Smith, 1997). Route *et al.* (2006) also found that the conventional methods of propagation in Begonia are problematic due to rapid occurrence of diseases. When vegetative propagated plants are systemically infected with pathogens, they are readily passed from one vegetative generation to the next, and it is possible that the entire population of a particular clonal variety can be lost (Simmonds, 1992). Most of *Begonia* x *hiemalis* Fotsch. are very susceptible to bacterial leaf spot (*Xanthomonas begoniae*). *Begonia* x *hiemalis* Fotsch. and *Begonia cheimantha* exhibit varied levels of susceptibility to powdery mildew (*Erysiphe cichoracearum*). Hakkaart and Versluijs (1983) and Westerhof *et al.* (1984) have found that bacterial leaf spot and blight (*Xanthomonas begoniae*) can cause significant problems in greenhouse production of Elatior Begonias, but this can be eliminated by meristem culture of adventitiously produced buds. Thus, Route *et al.* (2006) suggested that plant cell culture technique is an alternative method for mass cloning of Begonia plants and also overcome the problems occurring in the conventional propagation.

Another problem is the variation between individual plants, which has become more apparent since clonal selection in *Begonia* x *hiemalis* Fotsch. gained importance (Westerhof *et al.*, 1984). Mikkelsen and Sink (1978) found that the mutation rate in tissue-cultured Begonias was very low, but Simmonds and Werry (1987) identified that this study involved small populations. Therefore, Westerhof *et al.,* (1984) suggested that the variations of *in vitro* cultured clones should be compared with the variations of the same clone propagated conventionally. In a larger study, one cycle of micropropagation of *Begonia* x *hiemalis* Fotsch. gave nearly uniform offspring, but on the second and third cycles of *in vitro* multiplication, variation increased (Westerhof *et al*., 1984).

1.2 PLANT TISSUE CULTURE TECHNIQUE

In principle, plants can be propagated in two ways i.e. vegetatively (asexual, also called cloning) and generatively (sexual, by seeds) (Pierik, 1987). Under certain conditions generative propagation may be impossible and some plants cannot undergo this type of propagation. Thus, vegetative propagation especially through tissue culture technique could overcome the incapability of the plants to undergo generative propagation. Propagation of selected plant through tissue culture is called micropropagation (Phillips *et al*., 1995), which is also known as vegetative propagation.

In vitro culture is one of the key tools of plant biotechnology that exploits the totipotency nature of plant cell (Route *et al.*, 2006) and it can be employed for large-scale propagation. *In vitro* micropropagation procedure offers an efficient means of maintaining hybrid lines and allow for very rapid multiplication of new hybrid cultivars for early introductions (Simmonds, 1992). The use of tissue culture technique for commercial propagation of ornamental plants is a stepping-stone from the normal breeding. Some plants are now being commercially propagated using micropropagation techniques that are often looked upon as being practical for propagation of those plants, which are difficult to propagate by conventional methods. The procedures of plant tissue culture have developed to such a level that any plant species can be regenerated *in vitro* through several methodologies (Tisserat, 1985). This propagation is indeed applicable and beneficial to the "difficult to

propagate" species, and equally important, it may offer economic advantages for some species which are considered relatively "easy to propagate".

Thus, micropropagation seems to be the most promising method for the large-scale production of plantlets for use as pot plants (Pederson *et al*., 1996). Apart from that, micropropagation will also reduce the growth rate of the plant from their normal generative propagation. This technology involved the production of new plants from very small amount of tissues. The tissue or organ that is removed from a plant and placed into culture is called the explant. Under appropriate conditions, each explants will permit the rapid production of large numbers of identical plants.

Generally, the application of plant cell and tissue culture technique which is commonly known as *in vitro* cloning can also be divided into several procedures, including meristematic cultures, vegetative explants cultures, callus induction, suspension cultures, direct and indirect somatic embryo induction, synthetic seeds production, *in vitro* flowering, *in vitro* mutation breeding, protoplast and also somatic hybridization process. Some of these techniques would selectively apply to selected plants to overcome generation incapability of the plants.

Modern technique of propagation through tissue culture technique has been developed to meet the demand of the horticultural industries including nursery industries. For pot plant production, the priority is to obtain early, synchronized and profuse flowering, together with a compact and homogenous plant size, rather than continuous flowering (Pederson *et al*., 1996). Pederson *et al.* (1996) also identified that for pot plant production to be successful, an efficient method for flower induction in small plantlets is thus required. Thus, the application of plant tissue culture technique is always required. The technology is widely applied in both research and development of improved crops (Maliro and Lameck, 2004). Route *et al.* (2006) reported that about 156 ornamental genera were propagated through tissue culture in different commercial laboratories worldwide.

Regeneration in plant tissue culture will be successful by maintaining various factors involved, including media factors and environmental factors. The media factors include media constituents, macronutrients, micronutrients, vitamins, amino acids, carbon source, complex nutritive mixtures, gelling agents, activated charcoal, plant growth regulators, and pH of the medium. Environmental factors on the other hand are the culture conditions under which explants are maintained. The environmental factors involved include the temperature and illumination of the culture room, agitation process and incubation period of the cultures. Raven and Johnson (2001) reported that the growth permitted due to the response to the environment. The success or failure of the cultures can be determined by both factors. Previous researches have showed that media factors and environmental

factors need to be maintained to obtain morphogenesis in plant tissue culture. By studying selected protocols derived from previous experiments, efficient and reliable vegetative propagation through *in vitro* cloning can be successfully carried out. Previous research showed that, if the growth regulators are appropriately balanced, a rapid rate of shoots proliferation may be produced within a short period of time i.e. from 8 to 12 weeks of cultures.

1.2.1 Source of Explants

The micropropagation and regeneration of plants *in vitro* can be performed by using the organs or parts of organs or tissues of the plants which is known as explants. Thus, selection of appropriate donor tissue is the most critical aspect affecting the performance of micropropagation systems and the physiological age of explant influences the type and extent of morphogenesis (Simmonds, 1992). Competent shoot regeneration was influenced also by the physiological state of the donor plant (Becerra *et al.,* 2004). Practically any part of the plant can regenerate plantlet provided the explant is obtained at the proper physiological stage of development (Tisserat, 1985). Intact plants grown under controlled conditions prior to *in vitro* culture will ensure healthier explants and uniform response to the first reaction (Debergh and Maene, 1981) in plant tissue culture protocol especially during the regeneration process. Aseptic seedling is the most suitable explants normally used to overcome contamination problem. Immature tissues and organs are invariably more morphogenetically response *in vitro* than mature tissues and organ (Tisserat, 1985).

Becerra *et al.* (2004) had identified that the highest *de novo* shoot production frequency was achieved in explants from 2-month-old plants.

1.2.2 Medium Selection and Preparation

The composition of the culture medium is an important factor in the successful establishment of a tissue culture (Reinert and Bajaj, 1977). A defined nutrient medium consists of a balanced combination of several components i.e. macronutrient and micronutrient elements, vitamins, amino acids, carbon source (sugars), organic growth factors and growth regulators, agar or gelrite and water. The media allows the cultures to maintain their normal biochemical and physiological processes during use of the technologies listed in plant tissue culture procedures (Maliro and Lameck, 2004). The Murashige and Skoog (1962) (MS), Linsmaier and Skoog (1965) (LS) or Gamborg's, B5 are the most widely used salt compositions, especially in plant regeneration and have been proven effective for the growth of a variety of dicotyledonous and monocotyledonous plants. Media can be prepared either semi-solid or liquid and the difference between both types of media depend on the addition of agar prior to autoclaving.

The macronutrients consists of magnesium, calcium, phosphorus, sulfur, nitrogen, potassium and iron, cuprum, manganese, cobalt, molybdenum, boron, iodine, nickel, chlorine and aluminum are considered as micronutrients. The need for macronutrients is much larger as compared to micronutrients and is reflected by the concentrations of these

elements in the medium. Other than macro and micronutrients, plants tissue culture medium widely use vitamins such as thiamine (vitamin B1), niacin (vitamin B3), pyridoxine (vitamin B6), and myo-inositol (a member of the vitamin B complex).

Sucrose is the common carbon source used in plant, cell, tissue and organ culture media (Hazarika, 2003) and appropriate level of sucrose in the media is very important to compensate the deficiency of photosynthesis. Sucrose is a very important component in any nutrient medium and it's addition for *in vitro* growth and development, because photosynthesis is insufficient, due to the growth taking place in conditions unsuitable for photosynthesis or without photosynthesis (in darkness) (Pierik, 1987). Ebrahim (2004) identified that green cells and tissues are not sufficiently autotrophic *in vitro*, because of limiting conditions for photosynthesis (e.g., light, CO_2, etc.) and the effect of sucrose concentration on plant micropropagation led to inconsistent results which is also dependent upon the explant types and culture conditions.

1.2.3 Sterilization Procedures

The application of plant cell, tissue and organ culture require sterilization techniques (Gamborg and Phillips, 1995). The contaminants may also give out metabolic wastes which are toxic to plant tissues (Bhojwani and Razdan, 1996). Several techniques will be identified to represent the suitable sterilization procedures of selected plant to overcome

contamination. Sterilization can be carried out aiming for physical destruction of micro-organisms by using dry hot air, steam or irradiation (uv light or gamma irradiation). Some sterilizing compounds such as ethylene oxide, alcohol, sodium hypochlorite or antibiotics have been used to destruct the chemical of microorganism. Physical removal of microorganism can also be done by filtration or washing. Basically, three types of sterilization have been identified for obtaining successful tissue culture procedures. These include the preparation of sterile media and instruments, preparation of sterile or axenic explant materials and maintenance of aseptic conditions of the cultures. Once the three categories were established, tissue culture procedures can be done aseptically in a laminar air flow. A laminar air flow cabinet is one in which the air is sucked from the outside, first being filtered through very fine filters before reaching the table top of the inoculation cabinet (Pierik, 1987) for obtaining successful tissue culture procedures to overcome contamination problems.

Plant tissue culture media is normally rich in sucrose and other organic nutrients that can support organogenesis in plants but also the growth of many microorganisms (like bacteria and fungi) (Bhojwani and Razdan, 1996). To overcome and prevent contamination in media preparation, sterilization should be done thoroughly. Sterilization of nutrient media can be done in an autoclave (large pressure cooker), less often by filtration and seldom by irradiation (Pierik, 1987). The container with the medium should be properly closed and autoclaved at 121º C, 105 kPa, for 20 minutes. Pierik (1987) also identified that good sterilization relies on time, pressure, temperature and volume of the object to be sterilized.

The sterilized nutrient media should be stored in a sterile box that has previously been disinfected with 96% alcohol (Pierik, 1987).

Some of the plant growth regulators such as Giberellic acid (GA_3), zeatin, abscisic acid (ABA), urea, certain vitamins, pantothenic acid, antibiotics, colchicines, plant extracts and enzymes used in tissue culture are thermolabile (Bhojwani and Razdan, 1996). These compounds should not be autoclaved and filter-sterilization is often used if a thermolabile substance is needed in a nutrient medium. This can be done by using a cellulose acetate or cellulose nitrate filter with a pore diameter of 0.22 micron.

Surface of plant parts carry a wide range of microbial contaminants. The presence of any contaminant will interfere with the growth of explant or cultures and fungal or bacteria explant contamination in plant cultures is usually detectable 1-14 days after planting (Tisserat 1985). Therefore, sterilization or disinfection of tissues is necessary in order to eradicate surface microorganism.

In order to disinfect plant tissues, 5-50% (v/v) commercial bleach Clorox (sodium hypochlorite), 70% (v/v) alcohol and a few drops of Tween 20 (Polyoxyethylene sorbitan monolaurate) can be used in sterilization technique. Pierik (1987) has suggested that sterilizing plants for a few seconds in alcohol is not sufficient to kill all micro-organism and after this they are usually treated with hypochlorite. Tisserat (1985) found that most

commonly, a dilute solution of sodium hypochlorite (0.25-2.63%) (v/v) is used as a disinfectant and Tween 20 is an emulsifier which is added at the rate of 1 drop per 100 ml of solution.

The instruments including forceps and scalpels were sterilized by dipping them into hot beads at 250º C and allowed to cool. Glassware, empty test tubes, empty flasks, petri dishes, filter paper and distilled water can be sterilized using an autoclave at 121º C, 105 kPa, for 20 minutes. The bottles and glassware should not be too tightly packed and their tops should be loosen during autoclaving.

1.2.4 Incubation and Culture Conditions

Plant tissue cultures grow and give different responses depending on the type of culture environment they are subjected to. The intensity, type and duration of light, temperature, oxygen or carbon dioxide and other gas concentrations and physical composition of the medium all play a role in morphogenesis of the culture (Tisserat, 1985). Generally, explants are established under 500-1 000 Lux illumination intensity using a 16 hours photoperiod which is usually supplied by cool-white or special plant growth fluorescent lamps. Later, plantlet development is enhanced by higher light intensities such as 5 000-10 000 Lux which will promote photosynthetic leaf development and growth of the plantlets

to establish the plant in the *in vivo* environment. However, the best condition to induce embryogenic callus is darkness. Thus, the influence of illumination intensity and different photoperiod could result in different responses by the culture *in vitro*.

The conditions and temperature under which *in vitro* cultures are maintained can determine the success or failure of the attempt and also affect growth and development. The optimal temperature for *in vitro* growth and development of *in vitro* cultures inside the culture room is generally 3-4 °C higher than *in vivo*. The temperature inside the culture room is usually maintained at around 25 ± 1 °C. Sometimes, a lower temperature (18 °C for bulbous species), or higher temperature (28-29 °C for tropical species) is chosen.

Other than illumination intensity and room temperature, the influence of pH in culture medium is also very important to maintain the micropropagation of the plants. In the majority of cases, the pH of a culture medium lies between 5.5 and 6.0 (Sarma *et al*., 1990) before sterilization. Skirvin *et al.* (1986) reported that there are significant differences between initial pH levels and pH levels following autoclaving particularly in the pH range of 5.7 to 8.5. Lower pH (below 4.5) and higher pH (above 7.0) generally stop growth and development *in vitro*. If the pH is too low, the auxin IAA and gibberellic acid become less stable, the agar become too sloppy, particular salts (phosphate, iron salt) may precipitate,

vitamin B1 and panthothenic acid become less stable and uptake ammonium ions is retarded (Pierik, 1987).

1.2.5 Acclimatization

During *in vitro* culture, plantlets grow under very special conditions in relatively airtight cultivation vessels, e.g., air humidity is higher and irradiance lower than in conventional culture (Pospisilova *et al*., 1999; Estrada-Luna *et al*., 2001). The use of airtight vessels decreases air turbulence that increases leaf boundary layers and limit the inflow of CO_2 and outflow of gaseous plant product from the vessels. The plants that develop under lower relative humidity have fewer transpiration and translocation problems *ex vitro* and persistent leaves that look like normal ones (Hazarika, 2003). These will lead to the abnormalities in morphology, anatomy and physiology of plantlets cultivated *in vitro*. Other than plantlets abnormalities, the environmental factors will also affect the physiological characteristics of the plantlets during acclimatization process. Plantlets undergo a rapid and extreme change in physiological functioning when they are removed from *in vitro* culture to soil mixture (Preece and Sutter, 1991). Many *in vitro* plantlets need gradual changes in environmental conditions to improve plant survival and speed up of acclimatization. After transferring the plantlets to the greenhouse, such abnormalities can be repaired.

One of the obstacles prior to acclimatization is the capability of *in vitro* true-to-type shoots in producing roots. Shoots which were directly rooted in soil showed higher percentage of survival in the field than those rooted under *in vitro* conditions (Hazarika, 2003). A successful tissue culture method of propagation must result in reestablishment in soil of a high frequency of the tissue culture-derived plants (Murashige, 1974). The successes of *in vitro* plantlets to undergo acclimatization process depend on their adaptation into their natural habitat. The plantlets that have been clonally propagated through tissue culture technique are usually commercialized.

Jeon *et al.* (2004) stated that *in vitro* grown plantlets are heterotrophic and have low photosynthetic efficiency. During acclimatization, they are forced to be an autotrophic plant, which could be improved by altering their environment growth conditions, such as increasing light intensity, humidity, air temperature or CO_2 concentration (Jeon *et al*., 2004). Acclimatization can be speed up by hardening of plantlets *in vitro* or after transplantation by decreasing the transpiration rate by antitranspirants including ABA, or by increasing photosynthetic rate by elevated CO_2 concentration (Pospisilova *et al*., 1999).

The greenhouse and field have substantially lower relative humidity, higher light level and septic environment that are stressful to micropropagated plants compared to *in vitro* conditions (Hazarika, 2003). Estrada-Luna *et al.,* (2001) found that within 6 days after

transplanting *in vitro* plantlets of chili anchor pepper (*Capsicum annuum* L. cv. *San Luis*) to the soil, the plantlets recovered from leaf wilting, reduced water relative content, increased in stomatal resistance and became photoautotrophic.

1.2.6 Micropropagation of Begonia

In recent years, tissue culture of *Begonia* x *hiemalis* Fotsch. has attracted attention in several countries (Westerhof *et al.,* 1984). Begonia was selected in this study to investigate the regeneration of Begonia *in vitro* and to develop a protocol for obtaining high yield. Attempts to obtain *in vitro* plants have been reported by many researchers using *Begonia* x *tuberhybrida* (Samyn *et al*., 1984), *Begonia* x *cheimantha* (Ringe and Nitsch, 1968; Fonnesbech, 1974a, 1974b) and *Begonia* x *hiemalis* (Appelgren, 1976; Mikkelsen and Sink, 1978).

Several authors have reported *in vitro* of *Begonia* x *hiemalis* Fotsch. work on organogenesis (Westerhof *et al*., 1984). Many previous publications dealt with *in vitro* propagation of Begonia through different types of explants such as leaf discs (Ringe and Nitsch, 1968; Roest *et al*., 1981; Cassells and Morrish, 1985), inflorescences (Pierik and Tetteroo, 1987), peduncles, petioles (Ringe and Nitsch, 1968; Cassells and Morrish, 1985; Castillo and Smith, 1997) and tubers (Samyn *et al*., 1984). However, petiole and leaf

segments are the most common explants used for studying nutritional and growth substance requirements for adventitious bud production (Simmonds, 1992).

1.3 DIFFERENT TYPES OF PLANT TISSUE CULTURE

1.3.1 Callus Induction

Callus is basically a more or less non-organized tumor tissue, which usually arises on wounds of differentiated tissues and organs (Pierik, 1987). The process of callus formation is termed callogenesis (Donnelly and Vidaver, 1988). Pierik (1987) reported that, two main approaches have been recognized for plant propagation through callus induction i.e. i) organogenesis where shoot or root are directly produced from callus and ii) somatic embrogenesis in which cells undergoing a complex process to form embryo-like structures. Callus of different species may vary in their textures, friability and coloration (Narayanaswamy, 1977). The formation of callus is not only depending on types of plants but also depending on different position of explants within the plant. All types of organs (roots, stems, leaves, flowers, etc.) and tissues can be used for starting materials for callus induction (Pierik, 1987). In general, callus can be classified as embryogenic and non-embryogenic. The embryogenic callus has potentialities to undergo one cycle of embryo formation and could regenerate new plantlets, whereas non-embryogenic callus cannot undergo regeneration cycle and normally die after certain incubation period.

Many other factors including growth regulators, genotypes, composition of nutrient media, physical growth factors etc are important for callus formation. The ability of individual plant to produce callus when placed into culture has been shown to be controlled by both

media composition (basal salts and growth regulators), and plant genotypes. The composition of the culture medium is important in determining the morphogenetic pathway (Gurel *et al*., 2001). The induction of callus is also influenced by both factors including internal and environmental factors. Chemical factors such as different types of hormones, sucrose content, pH, different types of gelling agar and different types of additives such as L-Proline, ascorbic acid, casein hydrolysate, yeast, coconut milk and 2,3,5-triioobenzoic acid (TIBA). Previous research found that 2,4-D is the best hormone for callus induction *in vitro*. Ekiz and Konzak (1997) and Pierik (1987) had observed that auxin and cytokinin have effects on callus induction and regeneration; varying their concentration in the medium, causes differences in amount, rate and growth pattern of explants. High temperature (22-28 °C) is normally advantageous for callus formation (Pierik, 1987).

Dixon (1985) stated that callus cultures may be derived from a wide variety of plant organs and the choice of explant in a suitable 'biological state' for callus initiation is very important i.e. young tissues are more suitable than mature ones. The cells in a callus may consist initially of a variety of cell types, sizes and shapes differing in their voculation, cell contents and wall thickening (Narayanaswamy, 1977). Pigmentation is considerably influenced by the level of dextrose, presence of soluble starch, nitrogen deficiency, temperature, light and exogenous auxin (Narayanaswamy, 1977). Cassells and Morrish (1987) have found that the morphogenetic potential of callus decreased on sub-cultures, for

instance, callus from the sixth sub-culture produced only one-tenth the numbers of adventitious shoots of the initial callus.

1.3.2 Suspension Culture

Cell suspension cultures created a new mean for producing cloning products in a large scale via liquid medium. The first step towards de novo regeneration is to establish callus or suspension cultures (Phillips *et al*., 1995b). Narayanaswamy (1977) found that the movement of the liquid medium facilitates fragmentation of tissue leading to smaller units and helps in their gaseous exchange. Cells in suspension can exhibit much higher rates of cell division than do cells in callus culture (Phillips *et al*., 1995b). Even though bioreactor could overcome the need for large scale production, however suspension culture is still needed. One of the limiting factors for the application of these technologies is the development of asynchronous embryos in most suspension cultures (Buyukalaca *et al*., 2003).

The incorporation of liquid-based medium for indirect somatic embryo induction could produce plants more efficiently than the conventional tissue culture. Direct regeneration through conventional tissue culture method could only produce less cloned plants whereas suspension cultures could produce plants more efficiently. Besides, rapid clonal propagation enhances mass propagation of selected plants, this technique is also a

prerequisite for genetic engineering work in plants. Thus, cell suspensions offer advantages when rapid cell division or many cell generations are desired, or when a more uniform treatment application is required such as during cell selection procedures (Phillips *et al*., 1995b). The synchronization of somatic embryo development in embryogenic callus in suspension cultures is a crucial step in taking advantage of somatic embryogenesis for high production potential and reduction of unit cost through automation (Tonon *et al*., 2001).

1.3.3 Somatic Embryogenesis

Plant regeneration from *in vitro* cultures can be obtained by embryogenesis and organogenesis (Filho and Hattori, 1997). Recently, somatic embryogenesis has attracted attention in plant biotechnology, because it provides useful systems to produce starting materials for the production of synthetic seeds and also for induction of transgenic plants. Somatic embryogenesis is the process by which somatic cells differentiate into somatic embryo (Von Arnold *et al*., 2002) through characteristic embryological stages without fusion of gametes (Shumann *et al*., 1995). All plantlets obtained through embryogenesis did not differ phenotypically from the parental clones (Stefaniak, 1994). The most distinctive characteristic of any embryoid is its anatomical discrete and closed vascular system (Schumann *et al*., 1995).

The first attempt on somatic embryogenesis induction was achieved by Steward and Reinert (1958) on *Daucus carota* cell suspension. After that, a large number of plant species have been reported to form somatic embryos in culture. Somatic embryogenesis is the most promising technique for plant multiplication, because of its high proliferation potential and the fact that the risk of chimeric plant development in this case can be minimized or eliminated (Stefaniak, 1994). The induction of somatic embryogenesis in cell cultures demonstrated the persistence of totipotency in cells. All somatic cells within a plant contain the entire set of genetic information necessary to create a complete and functional plant (Merkle *et al*., 1995). Genotype is well-established as an important factor influencing the embryogenic response *in vitro* (Brown *et al.,* 1995). The induction of somatic embryogenesis must then consist of the termination of a current gene expression pattern in the explant tissue, and its replacement with an embryogenic gene expression program in those cells of the explant tissue which will give rise to somatic embryos (Merkle *et al*., 1995). Embryogenic culture growth is generally associated with changes in the synthesis and mobilization of proteins, carbohydrates and lipids (Silviera *et al*., 2004).

The distinction between direct and indirect somatic embryogenesis is unclear. However, embryogenic callus is composed of proembrogenic masses. The somatic embryos differentiate directly from the epidermal cells. Basically, somatic embryogenesis is known as a multi-step process including the induction of embryogenesis, proliferation of embyogenic cultures, embryo development or prematuration of somatic embryos, embryo

maturation and embryo germination. The process involved various developmental stages of adventitious bipolar structures such as proembryo, globular, heart-shaped, torpedo-shaped and cotyledonary-stage which under the appropriate conditions, can develop functional shoot and root systems. It is generally accepted that somatic embryogenesis follows a developmental pattern similar to zygotic embryogenesis (Merkle *et al*., 1995).

In order to efficiently regulate the formation of plants via somatic embryogenesis, it is important to identify the suitable plant growth regulators, endogenous and exogenous factors that coordinate and regulate the embryo body. Briefly, Brown *et al.* (1995) indicated that the major components for culture media for somatic embryogenesis should provide a carbon and energy source, inorganic macro and micronutrients, some level of reduced nitrogen, organic additives and growth regulators, primarily auxin and cytokinin. Usually embryogenic callus is formed in medium containing auxin (Von Arnold, *et al*., 2002). However, in a medium containing 2,4-D, the tissue multiplication goes on but mature embryos do not appear (Razdan, 1993). Initiation and proliferation of embryogenic cultures mainly need auxin and often also cytokinin, while prematuration of somatic embryos do not need plant growth regulator in the medium. Maturation of somatic embryos can be obtained by culturing on medium supplemented with ABA or reduced osmotic potential. Activated charcoal is also reported to improve embryogenesis in carrot (Razdan, 1993).

1.3.4 Synthetic Seed Production

Synthetic seed technology is an alternative to traditional micropropagation for production and delivery of cloned plantlets (Brischia *et al*., 2002). Synthetic seeds are very useful for plants, which do not produce viable seeds. Artificial seeds offer the possibility of a low-cost, high-volume propagation system that will compete with true seeds and transplants (Redenbaugh *et al*., 1984). The potential uses of artificial seeds are numerous, including delivery of elite germplasm, hand-pollinated hybrids with reduced seed fertility, and genetically engineered plants with sterile or unstable genotypes (Janeiro *et al*., 1997). The actual form of synthetic seed (i.e., presence or absence of a synthetic seed coat, whether they are hydrated or dehydrated, quiescent or not, etc.) may vary depending on the specific crop application (Gray *et al*., 1995). Synthetic seed research has been extended to numerous plants, including *Asparagus cooperi* (Ghosh and Sen, 1994), orchids, *Camellia japonica* (Janeiro *et al*., 1997), *Daucus carota* (Patel *et al*., 2000), *Paulownia elongata* (Ipekci and Gozukirmizi, 2003), *Fragaria ananassa* Duch. (Lisek and Orlikowska, 2004) and others.

The production of synthetic seeds in clonal plant or plant tissue culture will normally controlled by different factors i.e. the source of propagule, the function of encapsulation media, encapsulation procedures, storage period of the synthetic seeds, germination and adaptation to the substrates. Various factors have been investigated to establish the production of synthetic seeds.

Prior to production of synthetic seeds, various factors need to be examined. The encapsulation of synthetic seeds are analogous to the seed coats and endosperms of normal seeds (Gray *et al*., 1995). In many respects, seeds are an ideal propagation system because they (1) contain substantial quantities of food reserves to support the growth and establishment of the young seedlings; (2) are dormant or quiescent to minimize respiration and maximize longevity; and (3) are covered with protective seed coat to allow handling and reduce pathogen attack (McKersie *et al*., 1995). The seed coat may provide physical protection to the propagule, other than carry nutrients, growth regulators, fungicides, antibiotics etc. The capsule could be alginate or similar product, which would maintain the hydration of the embryo, allowing temporary storage to enhance the germination and establishments of the seedling. The germination and survival of the plantlets is ultimate aim of producing the synthetic seeds.

The production of synthetic seeds by encapsulating somatic embryos or micro shoots requires establishment and mass production of somatic embryos or micro shoots prior to standardization of encapsulation of the synthetic seeds. In order to mass-produce the synthetic seeds, a large numbers of high quality somatic embryos or micro shoots with synchronous maturation is required.

Synthetic seeds are seen by many as means of overcoming normal seeds which are not genetically identical to their parents, harbor a number of pathogens which can be inadvertently spread from contaminated seed production fields. The technology has applications in a diverse range of plant species, as an aid in the breeding of hybrid varieties, as a system of storage of valuable genetic resources and as a means of large-scale multiplication (McKersie *et al*., 1995).

The synthetic seeds formation could be developed on three kinds of explants i.e. shoot buds, somatic embryos and hairy roots. However, functionally, somatic embryos have yet to match the convenience of seed in order to overcome a number of obstacles i.e. problems with mass production, encapsulation and uniformity, must be overcome before synthetic seed can be useful for most crops (Gray *et al.,* 1995).

The encapsulation media for optimizing seeds germination should contained growth regulators i.e. auxins, cytokinins and also sucrose. The cotyledonary type of somatic embryos would be beneficial for supplying food once the seeds germinated. A higher shoot formation frequency of the root was observed when the sucrose concentration in the beads was about 3.0% (Oozumi and Kobayashi, 1995).

Encapsulation of shoot tips in calcium alginate offers another space saving option for storage, that is, preservation in alginate beads at low, but above zero temperatures (Lisek and Orlikowska, 2004). Cold storage of encapsulated embryos is also important for post-storage survival and germination (Ipekci and Gozukirmizi, 2003). Ghosh and Sen (1994) also reported that the advantages of synthetic seeds include ease of handling and potential long-term storage. Since medium or long storage is one of the aims of synthetic seed production, their storage life is a critical parameter (Janeiro *et al*., 1997). Mass production of somatic embryos with same stage of embryos and followed by seed encapsulation will fulfill the global synthetic seed market demand.

1.3.5 *In Vitro* Flowering

The application of tissue culture technique can also be used for the potential of increasing the efficiency of *in vitro* flowering induction for selected plants. Flowering is a unique development event in plants that involves the transition of vegetative shoot apex to form either an inflorescence or a floral meristem, and followed by initiation and subsequent maturation of the floral organs (Sim *et al*., 2007). The reproductive stage, or flowering process, is one of the critically important stages in plant development and is vital for the completion of the life cycle and seed production (Ziv and Naor, 2006). The timing of the transition from vegetative growth to flowering is paramount important in agriculture, horticulture and plant breeding because flowering is the first step of sexual reproduction

(Bernier *et al*., 1993). Under natural growth, flower formation usually commences when a plant attains maturity (Virupakshi *et al*., 2002).

In vitro flowering has been reported in many plant species including *Withania somnifera* Dunal. (Saritha and Naidu, 2007), *Cymbidium ensifolium* var. *misericors* (Chang and Chang, 2003), bamboo (Lin *et al*., 2003 and Nadgauda *et al.,* 1997), Bishop's weed (Pande *et al.,* 2002), lily (Nhut *et al*., 2001), green pea (Franklin *et al*., 2000), cranberry (Serres and McCown, 1994) and others. However very limited reports are available in Begonia.

Previous studies showed that *in vitro* flowering induction was affected by various factors, which include the nature and age of the explant, the composition of medium, plant growth regulators and prevailing environmental conditions (temperature, irradiance and photoperiod). Reproduction in the green plant depends on the presence and action of a flowering hormone (Hamner and Bonner, 1938). Previous investigations also found that exogenous cytokinins significantly influences *in vitro* flowering in many plant species (Chang and Chang, 2003; Lin *et al*., 2003). Other than cytokinin, Ziv and Naor (2006) suggested that gibberellins (GA_3) and auxins are the most commonly plant growth regulators used for *in vitro* flowering. Ringe and Nitsch (1968) found that, other than auxin and cytokinin, adenine is very important for the formation of flower buds on floral stalk explants.

Different explants give different responses regarding *in vitro* flowering in selected species. Previous research showed that various types of explants were used to obtain flowers *in vitro* including vegetative and reproductive organs.

Evans (1971) reported that light, temperature and nutrient factor would affect the period of flowering. However, Hamner (1940) suggested that there is no absolute requirement for light since the plant can flower even in continuous darkness. Takimoto (1960) observed that, all the plants producing little chlorophyll managed to initiate flower buds independently of the light conditions. However, Singh *et al.* (2006) found that specific photoperiod with some darkness is essential for *in vitro* flowering in embryogenic cultures but in continuous light or in darkness did not initiate flower. Thus, these findings showed that different photoperiods will affect floral bud development *in vitro*.

Sucrose is known to be the main choice of carbon source for *in vitro* flowering studies (Rastogi and Sawhney, 1987). Takimoto (1960) also identified that the plants cultured on the sucrose-supplied medium did not require high intensity light for flower initiation. The plants receiving sugar may require only long dark periods for flower initiation, the applied sugar replacing the high-intensity light process (Takimoto, 1960). Singh *et al*. (2006) discovered that sugars are necessary carbon source for reliable induction and development of flowers and addition of sugar to the medium is necessary for induction of floral

stimulus. Flower bud differentiation was observed only when the sucrose concentration was at 30-60 g/l (Singh *et al*., 2006). However, previous research found that the frequency and efficiency of flower bud differentiation was higher in the presence of 40 g/l sucrose in the medium (Lin *et al*., 2003; 2004); (Singh *et al.,* 2006).

1.3.6 Genetic Stability in Micropropagated Plants

Another benefit of tissue culture is the potential of increasing the efficiency of mutagenic treatment for variation induction and rapid cloning of large population for selected variants. The main principle elements of plant breeding are dealing with the induction of variation. Subsequently, desired characteristics of new individuals can be induced from the breeding. Mutation breeding is normally induced by physical mutagens such as x- or gamma rays or classical chemical mutagen such as EMS (ethyl methanesulphonate).

Mutagenesis treatment normally caused genetic effects on plants *in vivo* and *in vitro.* Mutations normally observed includes the changes in type of inflorescence, flower size, color, flowering period, leaf structure, size and color. The initial cycle of regenerants from leaf explants gave nearly uniform offspring, but when this was followed by one or two further cycle of *in vitro* propagation, the variation increased (Simmonds, 1992). Apart from that, mutations would also change plant habit and their adaptation. Positive achievements of *in vitro* mutagenesis have been recorded in other species which high

potential for commodity. Westerhof *et al.* (1984) has thoroughly investigated the problem of phenotypic variation of *Begonia* x *hiemalis* Fotsch. clones regenerated by *in vitro* propagation.

1.4 THE OBJECTIVES OF THE PRESENT WORK

The main aim of the present research was to examine the regeneration and multiplication of *Begonia* x *hiemalis* Fotsch cv. *Schwabenland Red in vitro* (Figure 1.1). Various explants such as leaf segments, peduncles, petioles, and stems taken from intact stock plants were induced to initiate cultures. Stock plants which were bought from Sungai Buloh Nursery, Selangor has been used in this study in order to get enough explant sources for regeneration. Apart from investigation on regeneration, the optimum medium identified was used to obtain the best explants for regeneration. Different types of plant growth regulators were used in this study including BAP, NAA, TDZ and various types of other auxins and cytokinins. All the cultures were incubated in the culture room with 16 hours light and 8 hours darkness with a temperature of 25 ± 1°C. The results were observed after 8 weeks and the cultures were subcultured every 6 weeks interval. The *in vitro* plantlets obtained were used for callus induction, suspension culture, somatic embryogenesis, synthetic seed production, *in vitro* flowering studies and acclimatization process.

Preliminary studies on callus induction was done using MS medium supplemented with different concentrations of 2,4-D (0.1-1.0 mg/l), different combinations and concentrations of BAP and 2,4-D and also TDZ. Two different explants derived from *in vitro* plantlets were used to identify the optimum media for callus induction. Different types of callus were also identified in this study i.e. embryogenic callus and non-embryogenic callus.

Plate 1.1: The 6-month-old intact plant of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red.*

Embryogenic callus was also found to undergo direct somatic embryogenesis. Induction of direct somatic embryos from two different types of explants such as leaf and petiole segments were carried out using solid media. By using MS medium supplemented with different compositions and concentrations of plant growth regulators, production of direct somatic embryogenesis was observed. Subsequently addition of additives such as casein hydrolysate, L-Proline and TIBA onto MS optimum medium were also tested to optimize direct somatic embryogenesis induction. The effect of photoperiod was also investigated in the present study. In this study, a new protocol for direct somatic embryogenesis of Begonia was accomplished and was used in synthetic seed production in subsequent work (Chapter 5).

Production of optimum embryogenic callus was also aimed to initiate cell suspension cultures. Callus produced from leaf explants were proliferated for the production of indirect somatic embryos. By sub culturing the embryogenic callus into suspension culture, the development of somatic embryos were also examined prior to sub culturing into regeneration medium.

The production of synthetic seeds from the *in vitro* cultures such as from micro shoots and somatic embryos was also carried out in this research. The main aim of producing synthetic seeds is to develop a clonal propagation system especially for non-seed producing plants such as *Begonia* x *hiemalis* Fotsch. The ability of the synthetic seed to be

stored within certain period make it useful for germination and multiplication process at different times and places.

Although Begonia are known as hybrid and non-seeded plant, their ability to produce flowers are very impressive. With high quality of blooming and different colors will always attract people worldwide. The aim of this part of the experiment was to induce *in vitro* flowering from different types of reproductive explants such as peduncle segments and inflorescences. The production of *in vitro* flowering will be useful to cut short the period of flower production, thus shortening the life cycle and also for understanding the physiology during flowering.

The complete *in vitro* regeneration and ability to adapt to natural environment is very important to accomplish plant tissue culture techniques. By transferring plantlets into soil, the survival rate of the *in vitro* plants from regeneration process, direct and indirect somatic embryos induction, synthetic seed production and *in vitro* flowering induction were determined. Apart from that, the microscopic differences between *in vivo* and *in vitro* leaf tissues prior and after hardening process and different developmental phases of the somatic embryos were also identified using scanning electron microscopy (SEM).

1.5 EXPERIMENTAL DESIGN

Treatments were arranged in a completely randomized design with sub sampling. There were 10 sterile tubes per treatment with 2 explants each for every tube. The experiments were conducted twice. The number of explants with harvestable shoots was recorded after 8 weeks on regeneration or shoot elongation medium. Harvestable shoots were defined as those having a vertical axis with a discernable apex and at least two nodes (at least 1.5–2.0 cm in length). The percentage of callus per explant was obtained by dividing the area of explants that produced callus with the total area of original explants. Treatment means were compared using the standard error of the mean.

CHAPTER 2

REGENERATION OF *Begonia* x *hiemalis* Fotsch. *IN VITRO*

2.1 EXPERIMENTAL AIMS

The use of tissue culture technique for commercial propagation of ornamental plants is an alternative to the normal breeding. Organogenesis leads to root formation or to the production of unipolar adventitious buds, which develop into shoots that have to be rooted for plantlet formation (Schumann *et al*., 1995).

The regenerative ability of an explant is influenced by several factors such as the organ from which it is derived, the physiological state of the explant and its size (Bhojwani and Razdan, 1996). Many previous publications dealing with *in vitro* regeneration have been reported for Begonia, including the utilization of different types of explants such as leaf discs (Ringe and Nitsch, 1968; Roest *et al*., 1981; Cassells and Morrish, 1985), inflorescences (Pierik and Tetteroo, 1987), peduncles, petioles (Ringe and Nitsch, 1968; Cassells and Morrish, 1985; 1987a; 1987b; Castillo and Smith, 1997) and tubers (Samyn *et al*., 1984).

Begonia is a temperate plant and does not produce seeds. Begonia was selected in this study to investigate the regeneration ability *in vitro* and to develop a protocol for the high yield of plant production. The plant materials used were *Begonia* x *hiemalis* Fotsch. cv.

Schwabenland Red and the source of the plants obtained from nurseries in Sungai Buloh, Selangor.

The purpose of this study was to investigate the use of micropropagation protocols to assist in *in vitro* regeneration of *Begonia* x *hiemalis* Fotsch. The main factors being investigated in this study were the use of different intact plant tissues as explant sources, sterilization procedures and culture conditions, preparation and selection of appropriate growth regulator levels to achieve *in vitro* regeneration and finally the mass production of *in vitro* regenerants through suspension cultures.

Four different intact explants such as leaf disks, petiole, peduncle and stem segments were utilized to identify the best explant for *in vitro* regeneration of Begonia. By using healthy intact explants, sterilization protocol was also established to overcome contamination problems prior and during incubation period. Different stages of sterilization procedures were also carried out prior to culture process to reduce contamination problems. Different solvents such as Tween 20, different concentrations of Clorox (sodium hypochlorite) and also 70.0 % of alcohol were applied as sterilizing agents.

Apart from that, different types of hormones were also supplemented in the MS media to obtain optimum growth and *in vitro* regeneration of Begonia by using intact explants. Other than BAP, NAA and TDZ, some auxins and cytokinins were also supplemented to the MS media to obtain *in vitro* regeneration of Begonia. The optimum media for *in vitro*

regeneration of Begonia was also used in the suspension culture media. This was aimed to rapidly mass produce the shoots of Begonia which may be applicable for commercialization purposes. Different concentrations of sucrose, coconut water and different pH levels were also tested to identify the suitable media for this study. The outcome of this study will be beneficial for further plant tissue culture studies in selected Begonia.

2.2 MATERIALS AND METHODS

2.2.1 A Protocol for Regeneration of *Begonia* x *hiemalis* Fotsch. *in vitro*

In order to establish *in vitro* regeneration of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red*, a suitable and novel protocol was developed. The protocol was developed not only for the culture process, but including the sterilization technique for intact plants used in this experiment. By using different types of explants and cultured onto MS medium supplemented with different combinations of hormones, sucrose contents, different concentrations of coconut water and different pH, an optimum medium and best explant was identified. The protocol was employed until the emergence of the micro shoots and followed by subculture onto MS developmental media for maturation of the plantlets prior to acclimatization onto soil.

2.2.2 Explant Preparation and Culture Procedure

Long–day treatment of the stock plants at 25 ± 1°C promoted regeneration *in vitro*. To maintain the plants, they were kept in the incubation room at 25 ± 1 °C, with 16 hours light and 8 hours dark photoperiod. The plants were watered once in 2 days interval and were applied once a week with 20N-8.7P-16.6K fertilizer (1.25 g/l) to maintain their growth and health. The plants were sprayed once a fortnight interval with fungicide to maintain the health of the plants and protect the plants from being infected with fungus. The vigorously growing plants were used in this study. In the present work, four types of explants including leaf disks, peduncle, petiole, and stems segments derived from intact plants were

evaluated in order to observe the morphogenetic response of these different organs under the same *in vitro* conditions. The leaf explants were cut approximately 5.0mm x 5.0mm, whereas the petiole explants were cut into 5.0-10.0mm long. Each explant was cultured on selected medium aiming for optimum regeneration process.

2.2.3 Sterilization Technique

Intact plants of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red* purchased from Sungai Buloh, Selangor were maintained in the culture room. Five to seven medium-sized (3-6 cm in diameter) healthy explants were collected from the 6-month-old of stock plants. The explants were surface sterilized for 30 minutes under running tap water to remove any contaminants, followed by stirring in 500 ml distilled water containing 1.0 ml/l Tween 20 (Fisher, Pittsburgh, PA, USA) for 20-30 minutes. The explants were rinsed in 50.0% (v/v) Chlorox with 1.0 ml/l Tween 20 for 1 minute, followed by 3 rinses in sterile distilled water. Finally, the explants were rinsed in 70.0% (v/v) alcohol for 1 minute followed by 3 rinses in sterile distilled water. Each rinse lasted approximately one minute.

2.2.4 Comparison of Different Orientation of Explants

The intact explants were cultured on MS and modified ½ strength MS medium devoid of plant growth regulators. Four different types of explants were cultured in different orientations in this study. The leaves were placed in 3 different orientations including abaxial surface down, adaxial surface down and horizontally. The peduncles, petioles and

stems were placed in 2 different orientations such as horizontal basal cut surface down and vertical positions. The percentage of shoot formation from different explant positions were observed. All cultures were incubated in the tissue culture room supplied with standard photoperiod and temperature i.e. under 16 hours photoperiod at illumination of 1 000 Lux and temperature maintained at 25 ± 1 ºC.

2.2.5 Screening of Suitable Explants for Shoot Regeneration in Different Combinations and Concentrations of Different NAA and BAP

Different combinations and concentrations of NAA and BAP were used to obtain a good combination range between NAA and BAP (Sigma Chemical Co.) for shoot formation. By using five combinations of NAA and BAP, the explants from intact plants were cultured in the selected media. All media contained 3.0 % (w/v) sucrose and 0.8 % (w/v) technical agar. The pH was adjusted to 5.8 before autoclaving.

Leaf disks, peduncle, petiole and stem segments were cultured in the media supplemented with combinations of NAA and BAP. There were 43 treatments in this experiment, with factorial combinations of six levels of NAA (0.1, 0.5, 1.0, 1.5, 2.0 and 3.0 mg/l) and six levels of BAP (0.1, 0.5, 1.0, 1.5, 2.0 and 3.0 mg/l) with one control. Each treatment was repeated twice.

The explants were cultured for 8 weeks. The morphogenetic responses were observed 8 weeks after the initiation of the cultures and the numbers of regenerated shoots were scored after 12 weeks. The following are the list of different concentrations and combinations of NAA and BAP that were used in this study.

1. MS + 0.0 mg/l BAP + 0.0 mg/l NAA
2. MS + 0.1 mg/l BAP + 0.0 mg/l NAA
3. MS + 0.5 mg/l BAP + 0.0 mg/l NAA
4. MS + 1.0 mg/l BAP + 0.0 mg/l NAA
5. MS + 1.5 mg/l BAP + 0.0 mg/l NAA
6. MS + 2.0 mg/l BAP + 0.0 mg/l NAA
7. MS + 3.0mg/l BAP + 0.0 mg/l NAA
8. MS + 0.1 mg/l BAP + 0.1 mg/l NAA
9. MS + 0.5 mg/l BAP + 0.1 mg/l NAA
10. MS + 1.0 mg/l BAP + 0.1 mg/l NAA
11. MS + 1.5 mg/l BAP + 0.1 mg/l NAA
12. MS + 2.0 mg/l BAP + 0.1 mg/l NAA
13. MS + 3.0 mg/l BAP + 0.1 mg/l NAA
14. MS + 0.1 mg/l BAP + 0.5 mg/l NAA
15. MS + 0.5 mg/l BAP + 0.5 mg/l NAA
16. MS + 1.0 mg/l BAP + 0.5 mg/l NAA
17. MS + 1.5 mg/l BAP + 0.5 mg/l NAA
18. MS + 2.0 mg/l BAP + 0.5 mg/l NAA
19. MS + 3.0 mg/l BAP + 0.5 mg/l NAA
20. MS + 0.1 mg/l BAP + 1.0 mg/l NAA
21. MS + 0.5 mg/l BAP + 1.0 mg/l NAA

22. MS + 1.0 mg/l BAP + 1.0 mg/l NAA
23. MS + 1.5 mg/l BAP + 1.0 mg/l NAA
24. MS + 2.0 mg/l BAP + 1.0 mg/l NAA
25. MS + 3.0 mg/l BAP + 1.0 mg/l NAA
26. MS + 0.1 mg/l BAP + 1.5 mg/l NAA
27. MS + 0.5 mg/l BAP + 1.5 mg/l NAA
28. MS + 1.0 mg/l BAP + 1.5 mg/l NAA
29. MS + 1.5 mg/l BAP + 1.5 mg/l NAA
30. MS + 2.0 mg/l BAP + 1.5 mg/l NAA
31. MS + 3.0 mg/l BAP + 1.5 mg/l NAA
32. MS + 0.1 mg/l BAP + 2.0 mg/l NAA
33. MS + 0.5 mg/l BAP + 2.0 mg/l NAA
34. MS + 1.0 mg/l BAP + 2.0 mg/l NAA
35. MS + 1.5 mg/l BAP + 2.0 mg/l NAA
36. MS + 2.0 mg/l BAP + 2.0 mg/l NAA
37. MS + 3.0 mg/l BAP + 2.0 mg/l NAA
38. MS + 0.1 mg/l BAP + 3.0 mg/l NAA
39. MS + 0.5 mg/l BAP + 3.0 mg/l NAA
40. MS + 1.0 mg/l BAP + 3.0 mg/l NAA
41. MS + 1.5 mg/l BAP + 3.0 mg/l NAA
42. MS + 2.0 mg/l BAP + 3.0 mg/l NAA
43. MS + 3.0 mg/l BAP + 3.0 mg/l NAA

All cultures were incubated in the tissue culture room supplied with standard photoperiod and temperature i.e. under 16 hours photoperiod at illumination of 1 000 Lux and the temperature maintained at 25 ± 1 ºC.

2.2.6 Screening for Suitable TDZ Concentration for Shoot Regeneration

Different concentrations of Thiadiazuron (TDZ) (Sigma Chemical Co.) were used for shoot induction. Four different explants i.e. leaf, peduncle, petiole and stem explants were utilized for this experiment. There were 6 treatments of TDZ (0.1, 0.5, 1.0, 1.5, 2.0 and 3.0 mg/l) in this experiment. Each treatment was repeated twice. The explants were cultured for 8 weeks. All cultures were incubated in the tissue culture room supplied with standard photoperiod and temperature i.e. under 16 hours photoperiod at illumination of 1 000 Lux and the temperature maintained at 25 ± 1 ºC. The morphogenetic responses were observed after 8 weeks. The number of regenerated shoots were scored after 12 weeks.

2.2.7 Screening for Suitable Auxins and Cytokinins for Shoot Regeneration

In order to study the effect of different types of auxins and cytokinins on shoot regeneration *in vitro*, 4 different types of auxins combined with 4 different types of cytokinins were selected. The selected auxins including Naphthalene acetic acid (NAA), Indole-3-acetic acid (IAA), Indolebutyric acid (IBA) and 2,4-Dichlorophenoxyacetic acid (2,4-D) whereas the cytokinins were 6-Benzylamino purine (BAP), 2-isopentenylaminopurine (2iP), Kinetin and Zeatin. MS media supplemented with 1.0 mg/l auxin and 1.0 mg/l cytokinin were prepared in this study. Peduncle and stem segments were cultured in the MS

supplemented with auxin, cytokinin, 3.0% (w/v) sucrose and 0.8% (w/v) technical agar for 8 weeks. All cultures were incubated in the tissue culture room supplied with standard photoperiod and temperature i.e. under 16 hours photoperiod at illumination of 1 000 Lux and the temperature maintained at 25 ± 1 ºC. The morphogenetic responses were observed after 8 weeks. The number of regenerated shoots were scored after 12 weeks.

2.2.8 The Effect of Different Concentrations of Sucrose, Coconut Water and pH on Regeneration

The effect of different concentrations of sucrose on shoot induction was tested using two different explants i.e. peduncle and stem explants. Peduncle and stem segments were cultured in the MS supplemented with 1.0 mg/l BAP, 1.0 mg/l NAA, 3.0% (w/v) sucrose, 0.8% (w/v) technical agar and different concentrations of sucrose from 1.0% to 5.0% (w/v). Each treatment was repeated twice.

The preparation of coconut water was adapted from Dodd and Roberts (1995). The coconut water was filtered through several layers of cheesecloth. The filtrate was then boiled for approximately 10 minutes to precipitate the proteins. Then, it was cooled to room temperature, followed by decanting and filtering the supernatant through a fairly rapid qualitative filter paper. Peduncle and stem segments were cultured in the MS supplemented with 1.0 mg/l BAP, 1.0 mg/l NAA, 3.0% (w/v) sucrose, 0.8% (w/v) technical agar and different concentrations of coconut water from 0.05%, to 0.25% (v/v). Each treatment was repeated two times.

The effect of different pH on shoot induction was tested using two different explants i.e. peduncle and stem explants. MS supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA, 3.0% (w/v) sucrose and 0.8% (w/v) technical agar was used. The pH of the media was set starting from 4.3, 4.8, 5.3, 5.8, 6.3, 6.8 and 7.3. The explants were cultured for 8 weeks. All cultures were incubated in the tissue culture room supplied with standard photoperiod and temperature i.e. under 16 hours photoperiod at illumination of 1 000 Lux and the temperature maintained at 25 ± 1 ºC. The morphogenetic responses were observed after 8 weeks. The number of regenerated shoots were scored after 12 weeks. The morphogenetic responses were observed after 8 weeks and the numbers of regenerated shoots were counted after 8 weeks.

2.2.9 Induction of Multiple Shoots through Suspension Culture

Multiple shoots of *Begonia* x *hiemalis* Fotsch. were induced from young stems and maintained on MS medium fortified with 1.0 mg/l BAP and 1.0 mg/l NAA, 3.0% (w/v) sucrose and 0.8% (w/v) technical agar (Sigma Chemical Co.) under 16 hours photoperiod at illumination of 2 000 Lux. The incubation temperature was maintained at 25 ± 1 ºC as mentioned before.

The cell suspension cultures were established after the multiple shoot cultures were about 6 weeks old. Suspension cultures were developed by inoculating 0.5g of multiple shoot into MS liquid medium fortified with 1.0 mg/l Zeatin, 1.0 mg/l IBA with 3.0% (w/v) sucrose. The cultures were shaken at 100 rpm on rotary shaker under the same photoperiod,

illumination and temperature. The influence of different volumes of the medium on the growth of micro shoot in cultures shaken (in 250-ml Erlenmeyer flasks) at 100 rpm was examined. The volumes tested were 30, 50, 100 and 150 ml/flask.

2.2.10 Statistical Analysis

All data and variables were statistically analyzed using SPSS statistical package version 11. Values are presented as mean ± SE. One-way ANOVA and Multiple Range Analysis were done on all data, using 95% LSD intervals method.

2.3 RESULTS

Explants were subjected to several treatments and culture conditions including addition of plant growth regulators, different pH, different sucrose content and coconut water for promoting efficient plant regeneration *in vitro*. Basically, different culture conditions will promote different response to the regeneration process.

Micropropagation of Begonia involves the process of explants selection and induction, development of micro shoots and plantlets, followed by hardening process. These were examined starting from culturing the selected intact explants onto selected medium and followed by incubating them in the culture room for 8 weeks, subculturing micro shoots into development media for another 8 weeks and lastly acclimatizing the plantlets in the greenhouse aiming for growth and flowering purposes.

Plant growth regulators play an important role in micropropagation especially in inducing regeneration. Auxin and cytokinin combinations were required for regeneration of Begonia. Adventitious shoot regeneration from leaf, peduncle, petiole and stem explants of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red* was very encouraging on MS medium supplemented with combinations of BAP and NAA. Application of these plant growth regulators to four different explants resulted in 30.00–100.00% regeneration, whereas by using single auxin or cytokinin, the explants did not give any response.

Different concentrations of BAP and NAA were selected starting from 0.0, 0.1, 0.5, 1.0, 1.5, 2.0 to 3.0 mg/l. Statistical analysis was carried out to verify significant difference between four different explants in this study. The results showed that combination of 1.0 mg/l BAP and 1.0 mg/l NAA is the optimum medium for regeneration (Table 2.1, Plate 2.1).

Encouraging responses were also obtained in four different cytokinins (Kinetin, 2-iP, BAP and Zeatin). Based on overall observations, Zeatin was the most effective cytokinin, while IBA was more effective than IAA or NAA for *in vitro* regeneration. Optimum conditions for regeneration from four different explants (leaf, peduncle, petiole and stem) of Begonia were evaluated in terms of regeneration frequency and number of regenerated shoots per explant. The results showed that Zeatin and NAA in combination of 0.5–1.0 mg/l and 0.1 mg/l for leaf and stem explants, and 3.0 mg/l and 1.0 mg/l for root explants, respectively gave good response. Shoots regenerated *in vitro* were rooted in growth regulator-free medium and transferred to soil.

2.3.1 Comparison of Different Orientations of Explants for Regeneration

The leaf explants were placed in 3 different positions, including abaxial surface down, adaxial surface down and horizontal stands. The results showed that the leaf explants placed in position abaxial surface down gave high percentage of regeneration (28.00%) as compared with another two positions respectively i.e. 15.00% for vertical position and 17.00% for adaxial surface down (Fig. 2.1).

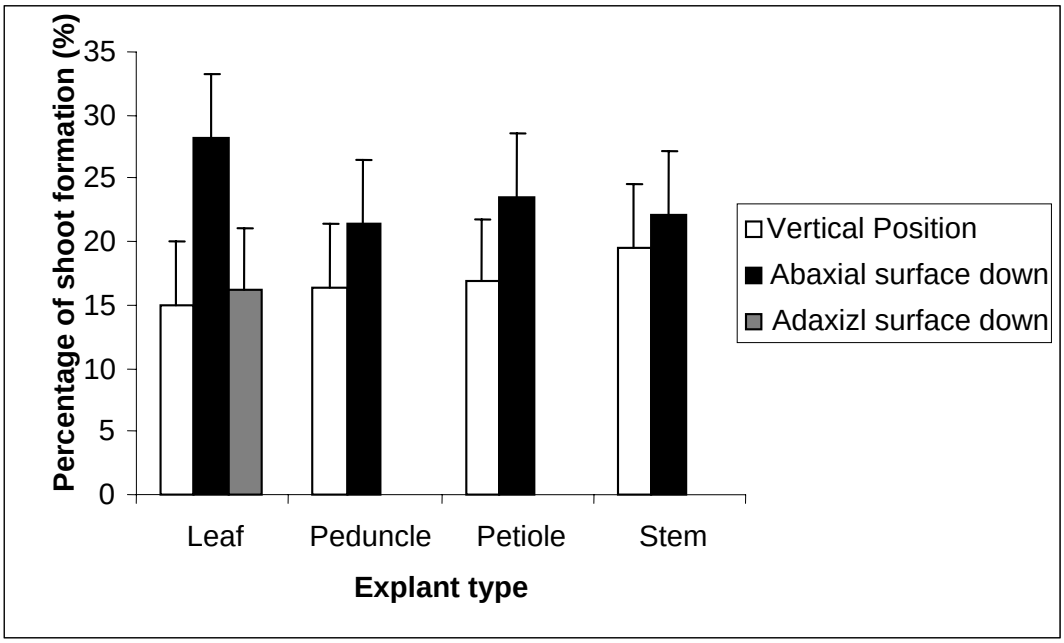


Fig. 2.1: The effect of different positions of explants on regeneration of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red* in the MS medium at 25 ± 1 ºC with 16 hours light and 8 hours dark.

The peduncles and petioles that were placed in two different positions, such as horizontal basal cut surface down and vertical positions, the horizontal position showed high percentage of regeneration (24.00%) as compared with vertical position (17.00%). There was a decline (from 17.00% to 15.00%) in the number of shoot formation with vertical position .The same results were obtained from petiole (17.00%) and stem explants (19.00%). Based on this experiment, two orientations of explants i.e. abaxial surface down and horizontal basal cut surface down were selected for further *in vitro* regeneration process.

2.3.2 The Effect of Different Concentrations and Combinations of BAP and NAA on Shoot Regeneration

Overall observation revealed that four different explants were capable of producing multiple shoots on hormone-supplemented medium (Table 2.1). From the screening experiment, the optimum medium for shoot regeneration was MS supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA which produced vigorous and normal shoots with dark green in color, whereas MS with 0.1 mg/l BAP in combination with 3.0 mg/l NAA was the best medium for root induction. From the results it could be interpreted that media supplemented with lower concentrations of BAP and NAA reduced the number of shoot formation.

Higher concentration of BAP (2.0 mg/l) and lower concentration of NAA (0.5 mg/l), increased the number of shoots produced (60.33 %)in leaf and peduncle explants but

reduced the sizes of the shoots. The numbers of shoots were reduced (13.7%) and rooting were optimum (56.7%) for stem explants, in the media supplemented with lower concentration of BAP (0.1 mg/l) and higher concentration of NAA (3.0 mg/l).

The cultures were observed every week for 8 weeks and observations were made prior to subculturing onto development medium. The explant sections began to swell after 2-3 weeks of culture in all media. Adventitious shoot buds and roots forming on the edges of swollen tissues became visible 3-4 weeks after culture initiation on culture media containing any amount of BAP (0.1-2.0 mg/l) and NAA (0.1-3.0 mg/l). Subsequently, the micro shoots developed into normal shoots afterr 4 weeks incubation.

All explants did not give any response in MS media without plant growth regulators and MS media supplemented with different concentrations of BAP alone. The explants did not undergo any differentiation and gradually turned brown and died on BAP supplemented medium, whereas on NAA supplemented medium, rooting was observed.

Another aspect to consider for plant regeneration is the source of explants. The source of explants cultured is important in determining the regenerative potential (Narayanaswamy, 1977). Four different explants were selected for *in vitro* shoot regeneration. The results indicated that leaf, peduncle, petiole and stem explants could regenerate into new plantlets *in vitro*. The most responsive tissue was petiole followed by leaf, peduncle and stem

explants. The percentage of shoot, the number of leaves and rooting were determined after 8 weeks of incubation.

All explants were placed vertically onto the surface of the solid media. The cut end of explants started to swell and produced multiple shoots at the cut ends. However, shoots produced from petiole and leaf explants grew vigorously and healthier compared to peduncle and stem explants (Table 2.1).

Table 2.1: Shoot and root organogenesis from different explants of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red* cultured on MS medium supplemented with NAA and BAP. The cultures were maintained at 25 ± 1 ºC with 16 hours light and 8 hours dark.

MS + NAA + BAP (mg/l)		Explant	Observations	*Explants with shoots (%) (Mean ± SE)	*Explants with roots (%) (Mean ± SE)
0	0	Leaf	-	0	0
		Peduncle	-	0	0
		Petiole	-	0	0
		Stem	-	0	0
0	0.1	Leaf	Necrotic	0	0
		Peduncle	Necrotic	0	0
		Petiole	Necrotic	0	0
		Stem	Necrotic	0	0
0	0.5	Leaf	Necrotic	0	0
		Peduncle	Necrotic	0	0
		Petiole	Necrotic	0	0
		Stem	Necrotic	0	0
0	1.0	Leaf	Necrotic	0	0
		Peduncle	Necrotic	0	0
		Petiole	Necrotic	0	0
		Stem	Necrotic	0	0
0	1.5	Leaf	Necrotic	0	0
		Peduncle	Necrotic	0	0
		Petiole	Necrotic	0	0
		Stem	Necrotic	0	0
0	2.0	Leaf	Necrotic	0	0
		Peduncle	Necrotic	0	0
		Petiole	Necrotic	0	0
		Stem	Necrotic	0	0
0	3.0	Leaf	Necrotic	0	0
		Peduncle	Necrotic	0	0
		Petiole	Necrotic	0	0
		Stem	Necrotic	0	0

Table 2.1: Continued

MS + NAA + BAP (mg/l)		Explant	Observations	*Explants with shoots (%) (Mean ± SE)	*Explants with roots (%) (Mean ± SE)
		Leaf	Normal shoots	No response	No response
0.1	0	Peduncle	Normal shoots	No response	No response
		Petiole	Normal shoots	No response	No response
		Stem	Normal shoots	No response	No response
		Leaf	Normal shoots	$15.33 \pm 1.33_{h}$	$5.00 \pm 0.00_{f}$
	0.1	Peduncle	Multiple micro shoots	$11.83 \pm 3.06_{h}$	$5.00 \pm 0.00_{e}$
		Petiole	Normal shoots	$33.33 \pm 4.54_{de}$	$6.67 \pm 0.63_{d}$
		Stem	Multiple micro shoots	$27.67 \pm 3.48_{d}$	$6.00 \pm 0.53_{g}$
		Leaf	Multiple micro shoots	$29.33 \pm 4.13_{e}$	0
	0.5	Peduncle	Multiple micro shoots	$85.33 \pm 1.92_{a}$	$0.33 \pm 0.00_{g}$
		Petiole	Multiple micro shoots	$65.33 \pm 4.52_{a}$	$0.22 \pm 0.00_{f}$
		Stem	Multiple micro shoots	$65.00 \pm 2.39_{a}$	$0.87 \pm 0.48_{h}$
		Leaf	Multiple micro shoots	$40.00 \pm 4.14_{d}$	$3.67 \pm 0.50_{g}$
	1.0	Peduncle	Multiple micro shoots	$38.67 \pm 4.87_{de}$	$1.07 \pm 0.53_{ef}$
		Petiole	Multiple micro shoots	$68.67 \pm 1.33_{a}$	$4.40 \pm 0.81_{d}$
		Stem	Multiple micro shoots	$23.33 \pm 3.33_{de}$	$0.67 \pm 0.45_{h}$
		Leaf	Multiple micro shoots	$26.00 \pm 3.20_{ef}$	No response
	1.5	Peduncle	Multiple micro shoots	$24.00 \pm 2.35_{f}$	No response
		Petiole	Multiple micro shoots	$22.67 \pm 2.06_{f}$	No response
		Stem	Multiple micro shoots	$22.67 \pm 2.06_{e}$	No response
		Leaf	Multiple micro shoots	No response	No response
	2.0	Peduncle	Multiple micro shoots	No response	No response
		Petiole	Multiple micro shoots	No response	No response
		Stem	Multiple micro shoots	No response	No response
		Leaf	Multiple micro shoots	No response	No response
	3.0	Peduncle	Multiple micro shoots	No response	No response
		Petiole	Multiple micro shoots	No response	No response
		Stem	Multiple micro shoots	No response	No response
		Leaf	Normal shoots/Rooting	No response	No response
0.5	0	Peduncle	Normal shoots/Rooting	No response	No response
		Petiole	Normal shoots/Rooting	No response	No response
		Stem	Normal shoots/Rooting	No response	No response
		Leaf	Multiple micro shoots	$25.00 \pm 3.78_{ef}$	$12.33 \pm 2.12_{d}$
	0.1	Peduncle	Multiple micro shoots	$18.33 \pm 2.11_{fg}$	$7.00 \pm 0.65_{e}$
		Petiole	Multiple micro shoots	$30.00 \pm 2.67_{e}$	$9.00 \pm 1.77_{e}$
		Stem	Multiple micro shoots	$26.33 \pm 2.82_{de}$	$20.67 \pm 2.84_{d}$

Table 2.1: Continued

MS + NAA + BAP (mg/l)		Explant	Observations	*Explants with shoots (%) (Mean ± SE)	*Explants with roots (%) (Mean ± SE)
		Leaf	Multiple micro shoots	$60.33 \pm 4.03_{ab}$	$5.00 \pm 0.00_{f}$
	0.5	Peduncle	Multiple micro shoots	$60.00 \pm 3.27_{bc}$	$5.00 \pm 0.00_{e}$
		Petiole	Multiple micro shoots	$25.00 \pm 0.00_{e}$	$5.00 \pm 0.00_{d}$
		Stem	Multiple micro shoots	$50.00 \pm 0.00_{b}$	$5.00 \pm 0.00_{g}$
		Leaf	Multiple micro shoots	$55.00 \pm 5.56_{c}$	$5.00 \pm 0.00_{f}$
	1.0	Peduncle	Multiple micro shoots	$45.00 \pm 3.27_{c}$	$5.00 \pm 0.00_{e}$
		Petiole	Multiple micro shoots	$43.33 \pm 2.95_{c}$	$5.00 \pm 0.00_{d}$
		Stem	Multiple micro shoots	$31.67 \pm 5.27_{d}$	$5.00 \pm 0.00_{g}$
		Leaf	Multiple micro shoots	$34.33 \pm 3.12_{d}$	0
	1.5	Peduncle	Multiple micro shoots	$40.00 \pm 3.27_{de}$	$3.40 \pm 0.53_{g}$
		Petiole	Multiple micro shoots	$32.33 \pm 5.36_{d}$	$0.67 \pm 0.67_{e}$
		Stem	Multiple micro shoots	$36.33 \pm 3.73_{d}$	0
		Leaf	Multiple micro shoots	$60.33 \pm 5.3_{ab}$	$2.00 \pm 0.82_{g}$
	2.0	Peduncle	Multiple micro shoots	$60.33 \pm 5.33_{bc}$	$3.67 \pm 1.33_{ef}$
		Petiole	Multiple micro shoots	$30.33 \pm 5.27_{d}$	$1.67 \pm 0.79_{e}$
		Stem	Multiple micro shoots	$42.33 \pm 5.43_{c}$	$2.40 \pm 0.82_{h}$
		Leaf	Multiple micro shoots	No response	No response
	3.0	Peduncle	Multiple micro shoots	No response	No response
		Petiole	Multiple micro shoots	No response	No response
		Stem	Multiple micro shoots	No response	No response
		Leaf	Normal shoots/Rooting	No response	No response
1.0	0	Peduncle	Normal shoots/Rooting	No response	No response
		Petiole	Normal shoots/Rooting	No response	No response
		Stem	Normal shoots/Rooting	No response	No response
		Leaf	Normal shoots/Rooting	$6.00 \pm 0.53_{i}$	$17.00 \pm 2.00_{c}$
	0.1	Peduncle	Normal shoots/Rooting	$6.33 \pm 0.60_{h}$	$12.33 \pm 2.12_{d}$
		Petiole	Normal shoots/Rooting	$10.33 \pm 1.14_{f}$	$14.00 \pm 2.14_{c}$
		Stem	Normal shoots/Rooting	$9.00 \pm 1.00_{g}$	$29.00 \pm 4.34_{c}$
		Leaf	Normal shoots/Rooting	$51.67 \pm 6.67_{c}$	$12.00 \pm 2.17_{d}$
	0.5	Peduncle	Normal shoots/Rooting	$58.33 \pm 5.27_{bc}$	$7.33 \pm 0.67_{e}$
		Petiole	Normal shoots/Rooting	$25.00 \pm 0.00_{de}$	$11.33 \pm 2.26_{c}$
		Stem	Normal shoots/Rooting	$47.33 \pm 1.18_{b}$	$6.00 \pm 1.56_{g}$
		Leaf	Normal shoots/Rooting	$68.67 \pm 4.77_{a}$	$18.67 \pm 4.10_{c}$
	1.0	Peduncle	Normal shoots/Rooting	$66.00 \pm 5.23_{c}$	$15.00 \pm 1.89_{d}$
		Petiole	Normal shoots/Rooting	$51.00 \pm 2.40_{b}$	$6.67 \pm 1.26_{d}$
		Stem	Normal shoots/Rooting	$51.67 \pm 4.80_{b}$	$22.00 \pm 3.96_{d}$

Table 2.1: Continued

MS + NAA + BAP (mg/l)		Explant	Observations	*Explants with shoots (%) (Mean ± SE)	*Explants with roots (%* (Mean ± SE)
		Leaf	Normal shoots/Rooting	$32.00 \pm 4.19_{e}$	$2.00 \pm 0.82_{f}$
	1.5	Peduncle	Normal shoots/Rooting	$32.00 \pm 4.19_{e}$	$14.00 \pm 1.77_{d}$
		Petiole	Normal shoots/Rooting	$26.33 \pm 4.23_{e}$	$5.33 \pm 1.33_{d}$
		Stem	Normal shoots/Rooting	$30.00 \pm 2.67_{d}$	$5.33 \pm 1.33_{g}$
		Leaf	Multiple micro shoots	$47.00 \pm 3.93_{c}$	$38.00 \pm 4.02_{a}$
	2.0	Peduncle	Multiple micro shoots	$47.00 \pm 3.90_{c}$	$7.67 \pm 1.37_{e}$
		Petiole	Multiple micro shoots	$41.67 \pm 3.98_{c}$	$7.00 \pm 0.65_{d}$
		Stem	Multiple micro shoots	$53.67 \pm 2.41_{b}$	$29.67 \pm 4.15_{c}$
		Leaf	Multiple micro shoots	$32.33 \pm 6.28_{e}$	$12.33 \pm 3.16_{de}$
	3.0	Peduncle	Multiple micro shoots	$41.33 \pm 5.33_{d}$	$6.67 \pm 1.87_{e}$
		Petiole	Multiple micro shoots	$27.33 \pm 3.38_{e}$	$6.00 \pm 0.53_{d}$
		Stem	Multiple micro shoots	$16.67 \pm 2.05_{f}$	$5.33 \pm 0.33_{g}$
		Leaf	Rooting	No response	No response
1.5	0	Peduncle	Rooting	No response	No response
		Petiole	Rooting	No response	No response
		Stem	Rooting	No response	No response
		Leaf	Multiple micro shoots	$73.86 \pm 5.03_{a}$	$22.33 \pm 2.17_{b}$
	0.1	Peduncle	Multiple micro shoots	$73.86 \pm 5.03_{b}$	$37.00 \pm 3.74_{ab}$
		Petiole	Multiple micro shoots	$23.00 \pm 4.30_{c}$	$24.00 \pm 1.00_{b}$
		Stem	Multiple micro shoots	$13.67 \pm 3.25_{f}$	$42.33 \pm 4.28_{b}$
		Leaf	Multiple micro shoots	$52.00 \pm 6.58_{c}$	$7.67 \pm 0.67_{de}$
	0.5	Peduncle	Multiple micro shoots	$52.00 \pm 4.58_{c}$	$5.00 \pm 0.00_{e}$
		Petiole	Multiple micro shoots	$36.67 \pm 4.19_{d}$	$4.33 \pm 0.96_{d}$
		Stem	Multiple micro shoots	$29.33 \pm 3.58_{d}$	$4.33 \pm 0.45_{g}$
		Leaf	Multiple micro shoots	$36.67 \pm 6.15_{de}$	$3.33 \pm 0.80_{f}$
	1.0	Peduncle	Multiple micro shoots	$36.67 \pm 6.15_{de}$	$2.00 \pm 0.65_{e}$
		Petiole	Multiple micro shoots	$41.33 \pm 4.24_{c}$	$3.00 \pm 0.65_{d}$
		Stem	Multiple micro shoots	$40.67 \pm 6.03_{c}$	$7.00 \pm 0.95_{f}$
		Leaf	Multiple micro shoots	$52.00 \pm 4.70_{c}$	$4.60 \pm 0.27_{f}$
	1.5	Peduncle	Multiple micro shoots	$52.00 \pm 4.70_{c}$	$22.33 \pm 3.41_{c}$
		Petiole	Multiple micro shoots	$15.67 \pm 2.23_{f}$	$7.00 \pm 0.95_{d}$
		Stem	Multiple micro shoots	$23.33 \pm 3.33_{d}$	$9.00 \pm 0.65_{f}$
		Leaf	Multiple micro shoots	$7.00 \pm 0.65_{i}$	$16.00 \pm 1.96_{c}$
	2.0	Peduncle	Multiple micro shoots	$7.00 \pm 0.65_{h}$	$11.67 \pm 1.93_{d}$
		Petiole	Multiple micro shoots	$11.33 \pm 0.91_{f}$	$9.67 \pm 1.72_{d}$
		Stem	Multiple micro shoots	$10.67 \pm 0.67_{g}$	$14.00 \pm 0.95_{e}$

Table 2.1: Continued

MS + NAA + BAP (mg/l)		Explant	Observations	*Explants with shoots (%) (Mean ± SE)	*Explants with roots (%) (Mean ± SE)
		Leaf	Multiple micro shoots	$16.00 \pm 3.62_{gh}$	$7.33 \pm 0.67_{e}$
	3.0	Peduncle	Multiple micro shoots	$16.00 \pm 3.62_{g}$	$6.00 \pm 0.72_{e}$
		Petiole	Multiple micro shoots	$12.67 \pm 1.18_{f}$	$5.00 \pm 0.49_{d}$
		Stem	Multiple micro shoots	$12.00 \pm 1.06_{fg}$	$5.00 \pm 0.95_{g}$
		Leaf	Rooting	No response	No response
2.0	0	Peduncle	Rooting	No response	No response
		Petiole	Rooting	No response	No response
		Stem	Rooting	No response	No response
		Leaf	Multiple micro shoots	$62.33 \pm 7.4_{bc}$	$25.00 \pm 1.09_{b}$
	0.1	Peduncle	Multiple micro shoots	$62.33 \pm 7.4_{bc}$	$33.00 \pm 4.30_{ab}$
		Petiole	Multiple micro shoots	$55.33 \pm 0.33_{b}$	$24.33 \pm 1.94_{b}$
		Stem	Multiple micro shoots	$52.67 \pm 3.93_{b}$	$29.33 \pm 1.65_{c}$
		Leaf	Multiple micro shoots	$56.00 \pm 6.30_{c}$	$10.33 \pm 1.65_{de}$
	0.5	Peduncle	Multiple micro shoots	$56.00 \pm 6.29_{cd}$	$5.00 \pm 0.00_{e}$
		Petiole	Multiple micro shoots	$45.00 \pm 2.67_{c}$	$5.00 \pm 0.00_{d}$
		Stem	Multiple micro shoots	$46.67 \pm 2.27_{bc}$	$8.00 \pm 0.24_{ef}$
		Leaf	Multiple micro shoots	$47.33 \pm 6.72_{cd}$	$8.00 \pm 0.65_{e}$
	1.0	Peduncle	Multiple micro shoots	$47.33 \pm 6.72_{d}$	$4.33 \pm 0.83_{e}$
		Petiole	Multiple micro shoots	$29.67 \pm 2.86_{e}$	$9.00 \pm 6.00_{d}$
		Stem	Multiple micro shoots	$25.00 \pm 3.59_{e}$	$2.33 \pm 0.40_{g}$
		Leaf	Multiple micro shoots	$65.67 \pm 3.68_{ab}$	$7.33 \pm 1.37_{de}$
	1.5	Peduncle	Multiple micro shoots	$65.67 \pm 3.68_{bc}$	$6.40 \pm 0.88_{e}$
		Petiole	Multiple micro shoots	$66.67 \pm 2.10_{a}$	$7.67 \pm 0.67_{d}$
		Stem	Multiple micro shoots	$22.00 \pm 2.92_{e}$	$2.67 \pm 0.50_{g}$
		Leaf	Multiple micro shoots	$37.33 \pm 3.00_{de}$	$12.00 \pm 0.65_{d}$
	2.0	Peduncle	Multiple micro shoots	$37.33 \pm 3.00_{de}$	$6.67 \pm 0.63_{e}$
		Petiole	Multiple micro shoots	$24.67 \pm 1.92_{ef}$	$7.00 \pm 0.65_{d}$
		Stem	Multiple micro shoots	$42.00 \pm 3.52_{c}$	$5.00 \pm 0.70_{g}$
		Leaf	Micro shoot/Rooting	$28.33 \pm 4.41_{ef}$	$7.33 \pm 0.67_{d}$
	3.0	Peduncle	Micro shoot/Rooting	$28.33 \pm 4.41_{e}$	$9.00 \pm 0.53_{d}$
		Petiole	Micro shoot/Rooting	$23.33 \pm 3.33_{e}$	$7.33 \pm 0.67_{d}$
		Stem	Micro shoot/Rooting	$25.67 \pm 4.28_{de}$	$7.86 \pm 0.65_{e}$
		Leaf	Micro shoot/Rooting	$28.33 \pm 4.39_{ef}$	No response
3.0	0	Peduncle	Micro shoot/Rooting	No response	No response
		Petiole	Micro shoot/Rooting	No response	No response
		Stem	Micro shoot/Rooting	No response	No response

Table 2.1: Continued

MS + NAA + BAP (mg/l)	Explant	Observations	*Explants with shoots (%) (Mean ± SE)	*Explants with roots (%) (Mean ± SE)
	Leaf	Micro shoot/Rooting	$11.00 \pm 1.96_{h}$	$36.33 \pm 4.10_{a}$
0.1	Peduncle	Micro shoot/Rooting	$11.00 \pm .196_{g}$	$53.33 \pm 4.16_{a}$
	Petiole	Micro shoot/Rooting	$18.33 \pm 2.05_{e}$	$36.33 \pm 3.22_{a}$
	Stem	Micro shoot/Rooting	$13.67 \pm 4.04_{f}$	$56.67 \pm 4.54_{a}$
	Leaf	Micro shoot/Rooting	$22.67 \pm 3.00_{fg}$	$20.00 \pm 2.63_{b}$
0.5	Peduncle	Micro shoot/Rooting	$22.67 \pm 3.00_{f}$	$28.33 \pm 3.64_{c}$
	Petiole	Micro shoot/Rooting	$34.67 \pm 3.32_{d}$	$21.67 \pm 3.83_{b}$
	Stem	Micro shoot/Rooting	$36.67 \pm 3.46_{d}$	$17.00 \pm 2.00_{d}$
	Leaf	Micro shoot/Rooting	$60.33 \pm 2.26_{b}$	$5.00 \pm 0.00_{f}$
1.0	Peduncle	Micro shoot/Rooting	$58.67 \pm 2.10_{cd}$	$5.00 \pm 0.00_{e}$
	Petiole	Micro shoot/Rooting	$48.00 \pm 2.62_{bc}$	$5.00 \pm 0.00_{d}$
	Stem	Micro shoot/Rooting	$29.33 \pm 4.07_{e}$	$5.00 \pm 0.00_{fg}$
	Leaf	Micro shoots	$26.67 \pm 4.13_{ef}$	$5.00 \pm 0.00_{f}$
1.5	Peduncle	Micro shoots	$29.33 \pm 4.22_{f}$	$5.00 \pm 0.00_{e}$
	Petiole	Micro shoots	$48.67 \pm 5.68_{bc}$	$5.00 \pm 0.00_{d}$
	Stem	Micro shoots	$26.67 \pm 4.24_{d}$	$5.00 \pm 0.00_{fg}$
	Leaf	Micro shoots	$12.00 \pm 2.17_{h}$	$5.33 \pm 0.59_{f}$
2.0	Peduncle	Micro shoots	$12.33 \pm 2.12_{g}$	$4.67 \pm 0.33_{e}$
	Petiole	Micro shoots	$14.33 \pm 2.38_{h}$	$5.00 \pm 0.00_{d}$
	Stem	Micro shoots	$11.00 \pm 1.96_{f}$	$4.33 \pm 0.45_{fg}$
	Leaf	Micro shoots	$33.33 \pm 4.71_{ef}$	$26.67 \pm 4.80_{b}$
3.0	Peduncle	Micro shoots	$36.33 \pm 4.77_{de}$	$24.00 \pm 3.32_{c}$
	Petiole	Micro shoots	$25.00 \pm 0.87_{e}$	$18.33 \pm 4.38_{b}$
	Stem	Micro shoots	$24.33 \pm 0.80_{de}$	$19.00 \pm 3.09_{d}$

* Mean values within a column by the same letters are not significantly different at the 0.05 level according to LSD test between the same explant.

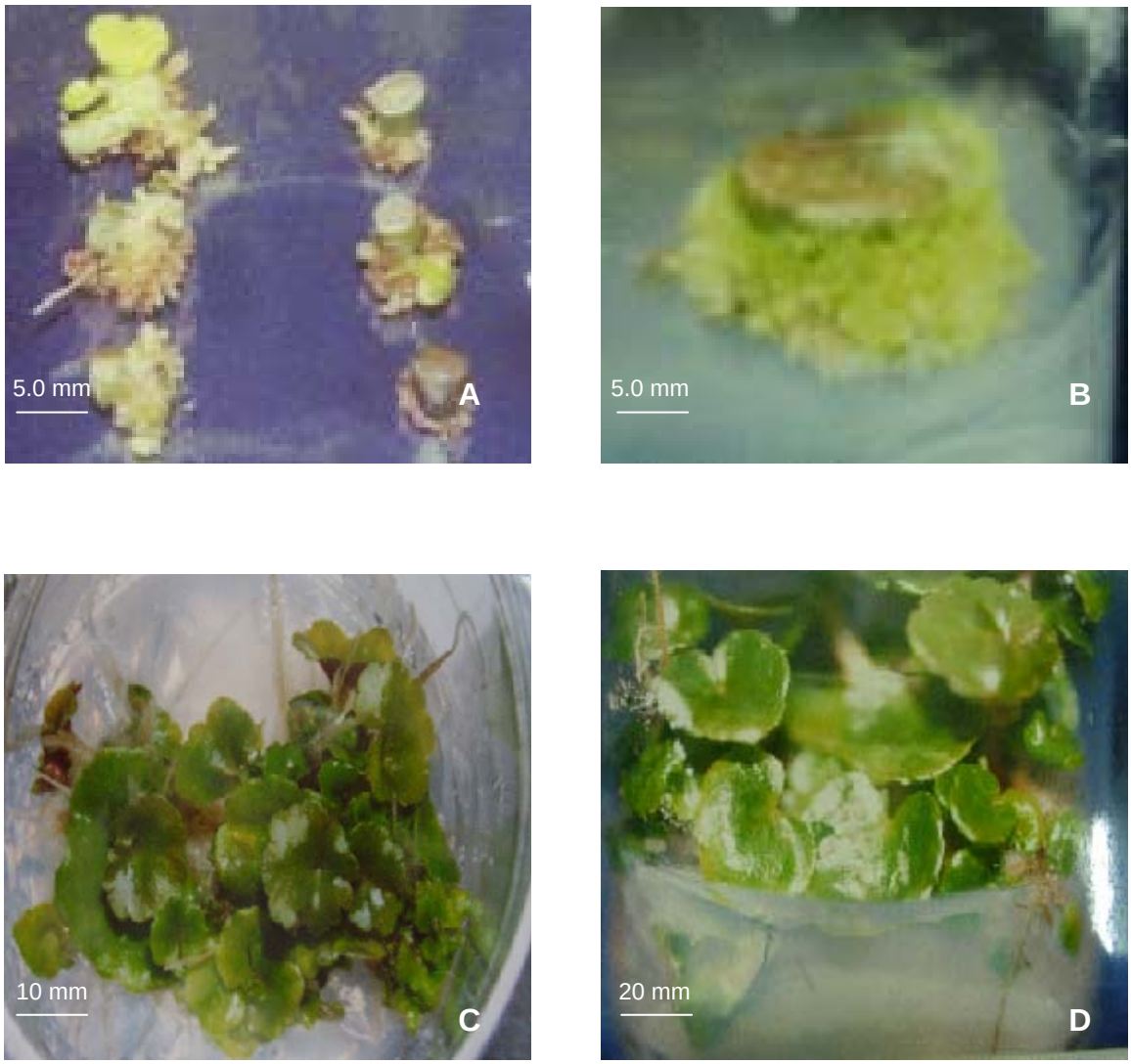


Plate 2.1: (A) Response of stem explants after being cultured on various orientations in MS basal medium. (B) Micro shoots formed from stem explants of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red* placed on MS medium supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA after 8 weeks of culture. (C) Multiple shoots formed from leaf explants of *Begonia* x *hiemalis* Fotsch. placed on optimum medium after 12 weeks and (D) 16 weeks of culture.

2.3.3 The Effect of Different Concentrations of TDZ on Shoot Regeneration

MS media supplemented with TDZ was effective in shoot regeneration in this study (Table 2.2). Shoot bud differentiation occurred directly from shoot segments within 2-3 weeks of culture on a TDZ supplemented medium and was free of any intervening callus formation. Well-developed shoots of about 20 mm in height were obtained in 4-5 weeks of culture. Shoot bud first appeared as small, green protuberances on the shoot segments and eventually developed into shoots (Plate 2.2).

From the results also showed that media supplemented with TDZ produced organogenesis response in all explants (Table 2.2). However, no rooting and callusing were observed in any concentration of TDZ. Morphological observations showed that the shoot buds emerged from each explant after two weeks being cultured onto media and the explants slightly enlarged before shoot formation. The percentage of shoot regeneration increased with the increasing concentration of TDZ from 0.1 mg/l to 0.5 mg/l. The optimal concentration for shoot regeneration was observed when the explants were placed on MS medium supplemented with 0.5 mg/l TDZ (Plate 2.2). A mean percentage of 70.00% to 72.67 % micro shoots was obtained from the optimum medium, MS supplemented with 0.5 mg/l TDZ. Higher concentration of TDZ (1-2.0 mg/l) increased the number of shoots produced (72.67-84.67%) but reduced the sizes of the shoots and abnormal shoots were produced from the explants (Plate 2.2A).

Table 2.2: Shoot formation from different explants of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red* after 8 weeks in culture on MS medium supplemented with various concentrations of TDZ. The cultures were maintained at 25 ± 1ºC with 16 hours light and 8 hours dark.

MS + TDZ (mg/l)	Explant	Observations	*Explants with shoots (%) (Mean ± SE)	*Percentage of Shoots/explant (Mean ± SE)	*Explants with roots (%) (Mean ± SE)
	Leaf	Abnormal shoots	100	73.33 ± 4.94 $_b$	-
	Peduncle	Abnormal shoots	100	73.33 ± 5.13 $_b$	-
0.10	Petiole	Abnormal shoots	100	73.33 ± 4.94 $_b$	-
	Stem	Abnormal shoots	100	72.00 ± 4.70 $_b$	-
	Leaf	Abnormal shoots	100	70.00 ± 6.76 $_b$	-
	Peduncle	Abnormal shoots	100	72.67 ± 3.84 $_b$	-
0.50	Petiole	Abnormal shoots	100	70.00 ± 6.76 $_b$	-
	Stem	Abnormal shoots	100	72.00 ± 5.36 $_b$	-
	Leaf	Abnormal shoots	100	84.67 ± 2.91 $_a$	-
	Peduncle	Abnormal shoots	100	62.00 ± 1.75 $_c$	-
1.00	Petiole	Abnormal shoots	100	84.00 ± 2.89 $_a$	-
	Stem	Abnormal shoots	100	83.33 ± 2.70 $_a$	-
	Leaf	Multiple micro shoots	100	82.67 ± 1.18 $_a$	-
	Peduncle	Multiple micro shoots	100	84.00 ± 1.31 $_a$	-
1.50	Petiole	Multiple micro shoots	100	82.67 ± 1.18 $_a$	-
	Stem	Multiple micro shoots	100	82.00 ± 1.07 $_a$	-
	Leaf	Multiple micro shoots	100	72.67 ± 1.07 $_b$	-
	Peduncle	Multiple micro shoots	100	72.67 ± 1.82 $_b$	-
2.00	Petiole	Multiple micro shoots	100	74.00 ± 1.90 $_b$	-
	Stem	Multiple micro shoots	100	83.33 ± 1.59 $_a$	-
	Leaf	Multiple micro shoots	100	72.00 ± 1.63 $_b$	-
	Peduncle	Multiple micro shoots	100	81.33 ± 1.41 $_a$	-
3.00	Petiole	Multiple micro shoots	100	72.67 ± 1.18 $_b$	-
	Stem	Multiple micro shoots	100	81.33 ± 0.91 $_a$	-

* Mean values within a column followed by the same letters are not significantly different at the 0.05 level according to LSD test between the same explant.

Plate 2.2: (A) The effect of different concentrations of TDZ and (B) sucrose on shoot regeneration of stem explants after 8 weeks of culture. (C) The effect of MS medium supplemented with 0.5 mg/l of TDZ on shoot regeneration of stem explants after 8 weeks of culture. (D) Shoot multiplication in the suspension cultures after 4 weeks incubation.

Among the four explants selected for regeneration of Begonia *in vitro,* it was found that peduncles, petioles and stems produced vigorous shoot compared to leaf explants. However, normal shoots were produced from leaf explants. Most shoots were green in colour and some were red in colour.

2.3.4 The Effect of Different Combinations of Auxins and Cytokinins on Shoot Regeneration

In this study, different types of auxins and cytokinins were selected to investigate the effect of different types of growth regulators on regeneration. Since in this study identified that the optimum regeneration medium was MS supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA, the same concentration of other auxins and cytokinins were applied in this study (Table 2.3).

The results showed that different types of growth regulators induced shoot regeneration except for 2,4-D. Combinations of 2,4-D with cytokinins in the medium produced callus in the explants and inhibited shoot production. From the screening experiment, the optimum medium for shoot regeneration was MS supplemented with 1.0 mg/l Zeatin and 1.0 mg/l IBA which produced vigorous and normal shoots with dark green in color (Table 2.3).

Table 2.3: Shoot formation from different explants of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red* after 8 weeks in culture on MS medium supplemented with various combinations and concentrations of auxins and cytokinins. The cultures were maintained at 25 ± 1 ºC with 16 hours light and 8 hours dark.

MS + 1.0 mg/l auxin + 1.0 mg/l cytokinin	Explant	Observations	*Explants with shoots/callus (%) (Mean ± SE)	*No. Shoots/ explant (Mean ± SE)	*Explants with roots (%) (Mean ± SE)
BAP+NAA	Peduncle	Normal green shoots	74.00 ± 4.17 $_{b}$	4.20 ± 0.47	17.00 ± 2.00 $_{a}$
	Stem	Normal green shoots	70.00 ± 2.56 $_{b}$	18.20 ± 2.49 $_{a}$	23.33 ± 3.99 $_{a}$
BAP+IBA	Peduncle	Abnormal greenish shoots	25.00 ± 4.80$_{e}$	2.67 ± 0.12	0
	Stem	Abnormal greenish shoots	38.33 ± 2.75 $_{b}$	6.60 ± 1.17 $_{b}$	0
BAP+2,4-D	Peduncle	Whitish nodular callus	84.00 ± 4.23$_{a}$	0	0
	Stem	Whitish nodular callus	50.67 ± 1.88$_{d}$	0	0
BAP+IAA	Peduncle	Normal green shoots	63.33 ± 3.47$_{c}$	0	0
	Stem	Normal green shoots	62.67 ± 5.11$_{c}$	0	0
ZEA+NAA	Peduncle	Green multiple shoots	28.33 ± 1.80$_{e}$	10.80 ± 0.74 $_{b}$	13.67 ± 2.04 $_{b}$
	Stem	Green multiple shoots	56.00 ± 4.45$_{d}$	9.60 ± 0.32 $_{b}$	14.00 ± 1.56 $_{b}$
ZEA+IBA	Peduncle	Small multiple shoots	70.67 ± 4.19$_{b}$	3.40 ± 0.45 $_{c}$	10.67 ±1.37 $_{b}$
	Stem	Normal multiple shoots	77.33 ± 2.28$_{a}$	6.87 ± 0.64 $_{b}$	7.00 ± 0.65 $_{c}$
ZEA+2,4-D	Peduncle	Yellowish nodular callus	17.67 ± 4.16$_{f}$	6.33 ± 0.81 $_{c}$	0
	Stem	Yellowish nodular callus	22.33 ± 3.55$_{f}$	4.53 ± 0.60 $_{bc}$	0
ZEA+IAA	Peduncle	Normal green shoots	53.33 ± 3.47$_{d}$	0	0
	Stem	Normal green shoots	52.67 ± 5.11$_{d}$	0	0
KIN+NAA	Peduncle	Normal green shoots	7.67 ± 1.81$_{h}$	5.60 ± 1.19 $_{c}$	16.0 ± 2.45 $_{a}$
	Stem	Normal green shoots	48.67 ± 6.39$_{d}$	5.00 ± 0.61 $_{bc}$	18.00 ± 1.07 $_{a}$
KIN+IBA	Peduncle	Green micro shoots	5.00 ± 0.00$_{h}$	20.73 ± 1.04 $_{a}$	0
	Stem	Green micro shoots	7.00 ± 6.39 $_{b}$	16.20 ± 1.41 $_{ab}$	0
KIN+2,4-D	Peduncle	Yellowish nodular callus	17.00 ± 1.60$_{f}$	20.53 ± 1.75$_{a}$	0
	Stem	Yellowish nodular callus	38.00 ± 7.49$_{e}$	21.27 ± 1.33$_{a}$	0

Table 2.3: Continued

MS + 1.0 mg/l auxin + 1.0 mg/l cytokinin	Explant	Observations	*Explants with shoots/callus (%) (Mean ±SE)	*No. Shoots/ explant (Mean ± SE)	*Explants with roots (%) (Mean ± SE)
KIN+IAA	Peduncle	Abnormal green shoots	$23.33 \pm 3.77_{e}$	0	0
	Stem	Normal green shoots	$10.67 \pm 1.08_{g}$	0	0
2-iP+NAA	Peduncle	Normal green shoots	$8.67 \pm 1.14_{h}$	$12.60 \pm 1.66_{b}$	15.00 ± 1.69
	Stem	Normal green shoots	$5.00 \pm 0.00_{h}$	$9.20 \pm 0.94_{b}$	23.67 ± 0.59
2-iP+IBA	Peduncle	Normal green shoots	$12.67 \pm 1.07_{g}$	$17.00 \pm 1.65_{a}$	0
	Stem	Normal green shoots	$7.67 \pm 1.08_{g}$	$13.40 \pm 1.67_{b}$	5.00 ± 0.00
2-iP+2,4-D	Peduncle	Yellowish callus	$64.67 \pm 6.87_{c}$	$11.27 \pm 0.46_{b}$	0
	Stem	Yellowish callus	$68.00 \pm 2.00_{b}$	$9.13 \pm 0.65_{b}$	0
2-iP+IAA	Peduncle	Normal green shoots	$16.33 \pm 1.79_{f}$	0	0
	Stem	Normal green shoots	$18.67 \pm 0.91_{f}$	0	0
		F (peduncle)	sig.	sig.	sig.
		F (stem)	sig.	sig.	sig.

* Mean values within a column followed by the same letters are not significantly different at the 0.05 level according to LSD test between the same explant.

2.3.5 The Effect of Different Concentrations of Sucrose on Shoot Regeneration

The effects of different concentrations of sucrose on shoot regeneration were examined using two different explants i.e. peduncles and stems. MS media supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA and different concentrations of sucrose starting from 1.0% (w/v) to 5.0 %(w/v) were used. Formation of micro shoots were observed in both explants within 3-4 weeks.

Table (2.4) shows that different concentrations of sucrose could produce shoots and roots. The results showed that culturing at different sucrose concentrations onto MS media had no effect on plant establishment, but influenced the plant quality. Overall observation indicated that lower concentration of sucrose (1.0%) would normally reduce the response of the explants. Apparently, high sucrose level (5.0%) was more stressful for shoots, which exhibited reduced green leaves and poor development. In MS medium supplemented with 10.0g/l sucrose, greenish micro shoots could be obtained. MS with 3.0% of sucrose was the optimum for subsequent *ex vitro* survival and growth.

As shown in Table (2.4), there is no significant difference between the mean percentages of shoot formation from peduncle and stem explants. The data also showed that there was significant difference between the percentages of shoot formation in optimum media and media supplemented with different concentrations of sucrose. For all these five different concentrations of sucrose, the optimum media supplemented with 4.0% (w/v) of sucrose

Table 2.4: The Effect of Different Sucrose Concentrations on Regeneration of Peduncle and Stem Explants of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red* on MS media supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA at 25 ± 1 ºC with 16 hours light and 8 hours dark.

SUCROSE % (w/v)	Observations		Explants with shoots (%)* (Mean ± SE)		Explants with roots (%)* (Mean ± SE)	
	Peduncle	Stem	Peduncle	Stem	Peduncle	Stem
1.0	Multiple green shoots and roots	Multiple green shoots and roots	42.50±4.03 $_{d}$	25.94±3.91 $_{d}$	22.50±3.82 $_{a}$	22.50±0.56 $_{a}$
2.0	Multiple green shoots and roots	Multiple green shoots and roots	47.50±1.93 $_{cd}$	38.75±4.55 $_{c}$	19.69±3.78 $_{a}$	19.69±2.24 $_{a}$
3.0	Multiple shoots and roots	Multiple shoots and roots	65.00±5.00 $_{b}$	64.06±6.02 $_{a}$	20.62±2.66 $_{a}$	20.62±3.77 $_{a}$
4.0	Multiple brownish shoots and roots	Multiple brownish shoots and roots	70.94±2.51 $_{a}$	66.88±3.82 $_{a}$	12.81±1.1 $_{b}$	12.81±2.13 $_{b}$
5.0	Multiple reddish shoots and roots	Multiple brownish shoots and roots	58.75±2.56 $_{bc}$	50.00±2.63 $_{b}$	19.81±1.18 $_{a}$	19.81±3.82 $_{a}$
		F	sig.	sig.	Not sig.	Not sig.

* Mean values within a column followed by the same letters are not significantly different at the 0.05 level according to LSD test between the same explant.

has a significantly higher mean score than the others but 3.0% (w/v) of sucrose produced greenish and vigorous micro shoots compared with others.

2.3.6 The Effect of Different Concentrations of Coconut Water on Shoot Regeneration

Generally, both explants (peduncles and stems) successfully formed shoots on MS supplemented with 1.0 mg/l BAP, 1.0 mg/l NAA, 3.0% (w/v) sucrose, 0.8% (w/v) technical agar and different concentrations of coconut water (Table 2.5). The results showed that the micro shoot formation were significantly different in different concentrations of coconut water. Root formation was also observed from various concentrations of coconut water and the root formation decreased when the concentration of coconut water increased. The leaf colour maintained green and MS media with 1.0 mg/l BAP and 1.0 mg/l NAA supplemented with 2.0% (v/v) of coconut water did not show any significant increase or effective medium for shoot regeneration compared to other concentrations. The means of 65.05 (v/v) and 70.94% micro shoots were obtained from the cultures contained 1.5% (v/v) and 2.0% (v/v) coconut water.

As shown in Table (2.5), there is no significance different between the mean percentages of shoot formation in peduncle and stem explants. However there is significant difference between the percentages of shoot formation in optimum media (MS supplemented with 1.0 mg/l BAP, 1.0 mg/l NAA, 3.0% (w/v) sucrose and 0.8% (w/v) technical agar) and media supplemented with different concentrations of coconut water. Amongst the five different

Table 2.5: The Effect of Different Concentrations of Coconut Water on Regeneration of Peduncle and Stem Explants of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red* on MS media supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA at 25 ± 1 ºC with 16 hours light and 8 hours dark.

COCONUT WATER % (v/v)	Observations		*Explants with shoots (%) (Mean ± SE)		*Explants with roots (%) (Mean ± SE)	
	Peduncle	Stem	Peduncle	Stem	Peduncle	Stem
0.05	Pale green shoots and rooting	Pale green shoots and rooting	42.59±1.12 $_{d}$	25.94±1.62 $_{c}$	22.50±1.27 $_{a}$	22.50±1.27 $_{a}$
0.1	Pale green shoots and rooting	Pale green shoots and rooting	47.50±1.5 $_{d}$	38.75±1.21 $_{c}$	19.69±1.10 $_{a}$	19.69±1.10 $_{a}$
0.15	Green shoots and rooting	Green shoots and rooting	65.00±2.00 $_{b}$	64.06±2.01 $_{a}$	20.62±1.63 $_{a}$	20.62±1.63 $_{a}$
0.20	Green shoots and rooting	Green shoots and rooting	70.94±1.04 $_{a}$	66.88±1.37 $_{a}$	12.81±4.46 $_{b}$	12.81±4.46 $_{b}$
0.25	Green shoots and rooting	Green shoots and rooting	58.75±1.25 $_{c}$	50.00±2.56 $_{b}$	19.81±1.45 $_{a}$	19.81±1.45 $_{a}$
		F	sig.	sig.	Not. sig	Not.sig.

* Mean values within a column followed by the same letters are not significantly different at the 0.05 level according to LSD test between the same explant.

concentrations of coconut water, the optimum media supplemented with 2.0% (v/v) of coconut water gave a significantly higher mean score than the other concentrations.

2.3.7 The Effect of Different pH on Shoot Regeneration

The effect of different pH on the regeneration of peduncle and stem explants of *Begonia* x *hiemalis* Fotsch. was investigated in the MS media supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA and cultured at 25 ± 1 ºC with 16 hours light and 8 hours dark. Generally, the results revealed that all treatments gave good response on regeneration from both explants i.e. peduncles and stems. As shown in Table (2.6), there was no significant difference between the mean percentages of shoot formation in peduncle and stem explants. The data also showed that there was no significant difference between the percentages of shoot formation in pH 5.8 and media with pH 4.3, 4.8, 5.3 and 6.3. However, there was significant difference between the percentages of shooting in pH 5.8 and media with pH 6.8 and 7.3. The optimum pH for regeneration was 5.8 which had a significantly higher mean compared to other concentrations.

Table 2.6: The Effect of Different pH media on Regeneration of Peduncle and Stem Explants of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red* cultured on MS media supplemented with 1 mg/l BAP and 1.0 mg/l NAA at 25 ± 1 ºC with 16 hours light and 8 hours dark.

pH	Observations		*Explants with shoots (%) (Mean ± SE)		*Explants with roots (%) (Mean ± SE)	
	Peduncle	Stem	Peduncle	Stem	Peduncle	Stem
4.3	Brownish shoots and rooting	Pale green shoots and rooting	48.13 ± 4.30$_a$	66.25 ± 2.21$_a$	28.75 ± 1.25$_a$	16.56 ± 2.08$_c$
4.8	Brownish shoots and rooting	Pale green shoots and rooting	31.88 ± 2.92$_c$	53.12 ± 2.18$_b$	17.50 ± 1.12$_b$	32.50 ± 1.12$_a$
5.3	Green shoots and rooting	Green shoots and rooting	36.88 ± 3.26$_b$	48.75 ± 2.56$_b$	20.00 ± 1.29$_b$	32.50 ± 1.44$_a$
5.8	Green shoots and rooting	Green shoots and rooting	37.50 ± 4.79$_b$	62.50 ± 3.93$_a$	11.25 ± 1.85$_c$	26.88 ± 1.51$_b$
6.3	Green shoots and rooting	Green shoots and rooting	36.25 ± 2.34$_b$	54.38 ± 3.98$_b$	9.38 ± 1.43$_c$	24.38 ± 1.28$_b$
6.8	Pale green shoots and rooting	Pale green shoots and rooting	35.62 ± 4.83$_b$	22.50 ± 5.02$_c$	1.56 ± 1.59$_d$	5.63 ± 1.40$_d$
7.3	Pale green shoots and rooting	Pale green shoots and rooting	36.88 ± 1.45$_b$	21.87 ± 3.65$_c$	0.94 ± 0.50$_d$	1.88 ± 1.63$_e$
		F	not sig.	sig.	sig.	sig.

* Mean values within a column followed by the same letters are not significantly different at the 0.05 level according to LSD test between the same explant.

2.3.8 Induction of Multiple Shoots through Suspension Culture

Liquid or suspension culture was more effective in shoot multiplication in this study. The influence of different volumes of medium starting from 30, 50 100 to 150 ml, on the multiplication and growth of micro shoots was determined. The results revealed that rapid regeneration was recorded within 3-4 weeks after incubation. The frequency of shoot regeneration and the number of shoots produced per flask increased with the increasing volume of the liquid media from 30 ml to 100 ml. The shoots clumped together but grew vigorously and the leaf sizes were about 0.5-1.0 cm. Based on Fig. (2.2), 100 ml of suspension produced high quantity of multiple shoots compared with other volumes. A mean of 188 shoots per flask was obtained from 100 ml of suspension medium after 4 weeks of incubation, whereas the mean number of shoots obtained from 150 ml suspension medium was only 75 shoots per flask.

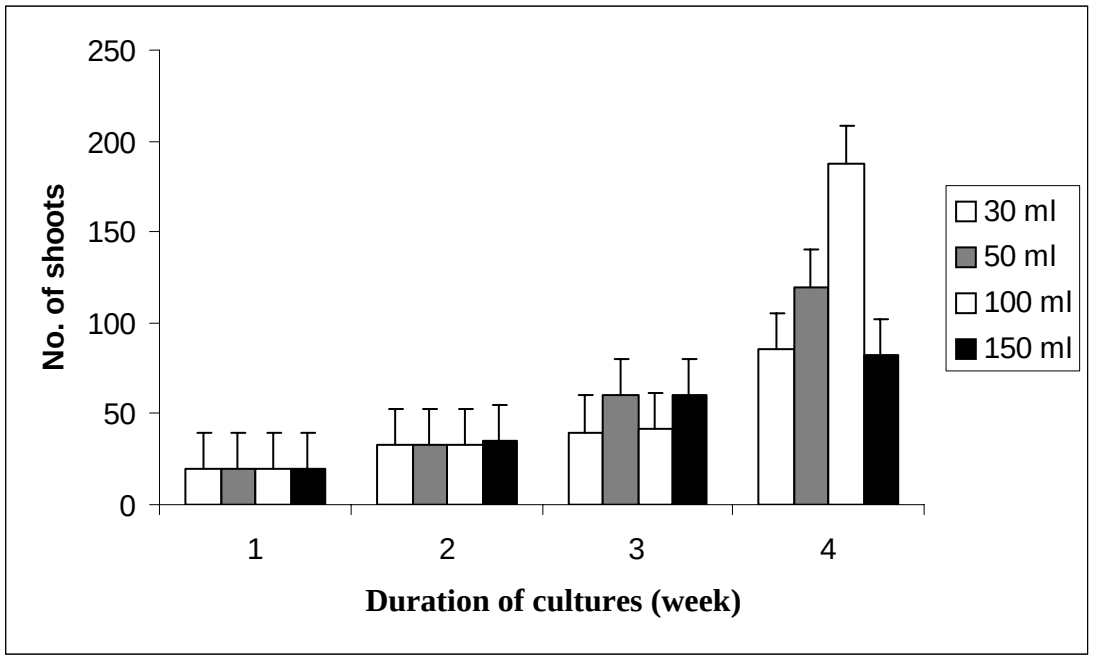


Fig. 2.2: The effect of different volume of suspension cultures on growth and multiplication of shoots on MS medium with 1.0 mg/l Zeatin and 1.0 mg/l NAA for 4 weeks.

2.4 SUMMARY

1. Leaf explants placed in position abaxial surface down and petioles placed in horizontal position gave the best percentage of shoot formation (24.0%) as compared with other positions when cultured on MS medium devoid of plant growth regulators.

2. MS medium supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA was the best media for shoot formation from the four different explants.

3. The best explant for shoot regeneration were leaf and petiole explants.

4. The frequency of shoot formation produced from leaf, petiole, peduncle and stem explants increased but the height of the shoots produced decreased with increasing concentration of BAP singly and BAP in combination with NAA.

5. The frequency of root formation from leaf, peduncle, petiole and stem explants increased with the decreasing concentration of NAA.

6. The best concentration for combination of auxin and cytokinin for shoot regeneration was 1.0 mg/l Zeatin and 1.0 mg/l IBA.

7. Generally TDZ also induced effective shoot formation similar with BAP in promoting shoot regeneration.

8. For optimum shoot formation, MS supplemented with 1.5% and 2.0% (v/v) of coconut water were most effective.

CHAPTER 3

CALLUS INDUCTION OF *Begonia* x *hiemalis* Fotsch. *IN VITRO*

3.1 EXPERIMENTAL AIMS

Callus tissue is basically known as an unorganized, undifferentiated growth or proliferation of cells. Callus formation can be successfully induced using different types of explants including vegetative and reproductive organs. Previous research showed that callus could be obtained from leaf, petiole explants (Cassells and Morrish, 1987b), pistil organs (An *et al*., 2004), seeds (Birsin and Ozgen, 2004), roots (Hoque and Mansfield, 2004) and other explants of Begonia.

The aim of this experiment was to find out the effects of explant sources, medium compositions and growth regulators in order to optimize the induction and selection of fast growing callus lines of *Begonia* x *hiemalis* Fotsch. cv. *Scwabenland Red*. Different concentrations and combinations of BAP and 2, 4-D were employed in this study. Both explants, i.e. leaves and petioles were used to obtain the optimum medium for callus production since from previous chapter it was found that the two explants were the responsive explants. Morphological characteristics of callus were also examined using double differential dyeing method adapted from Gupta and Durzan (1987) to study the different types of callus i.e. embryogenic and non-embryogenic callus. At the same time, the importance of some additives such as casien hydrolysate and L-Proline were used to

induce faster growth of callus. Subsequently, sufficient callus obtained were used for the production of direct and indirect somatic embryos in the following experiments.

3.2 MATERIALS AND METHODS

3.2.1 Explant Preparations

Intact plants of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red* (6-month-old) purchased from Sungai Buloh Nursery, Selangor were maintained in the culture room. Medium-sized healthy petioles were collected from the stock plants and used as explants to initiate cultures. The explants were surface sterilized for 30 minutes under running tap water for dehusking, followed by stirring in 500 ml distilled water containing 1.0 ml/l Tween 20 (Fisher, Pittsburgh, PA, USA) for 20-30 minutes. The explants were rinsed in 50% (v/v) Chlorox with 1 ml/l Tween 20 for 1 minute, and then rinsed 3 times in sterile distilled water. Finally, the explants were rinsed in 70% (v/v) alcohol for 1 minute and followed by 3 times in sterile distilled water. Each rinse lasted approximately one minute.

The explants were approximately cut into 5.0-10.0 mm segments and cultured onto MS medium supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA for shoot induction. The cultures were incubated and maintained in the culture room for 8 weeks. After 2 months of culture, the whole explants including micro shoots were separated from the media and subcultured onto MS fresh medium containing 3.0% (w/v) sucrose and 0.5 mg/l GA_3 for plantlet development. The plantlets were maintained in the culture room and callus cultures were established for subsequent experiments.

3.2.2 Callus Induction, Culture Media and Conditions

Leaf and petiole explants were used to study the induction of callus *in vitro* and the explants were obtained from the axenic cultures. The leaf explants were cut approximately 5.0mm x 5.0mm, whereas the petiole explants were cut into 5.0-10.0mm long. They were then cultured onto MS media supplemented with 2,4-D (0.1-3.0 mg/l) applied singly or with different combinations and concentrations of BAP (0.1, 0.5, 1.0, 1.5 and 2.0 mg/l) and 2,4-D (0.1, 0.5, 1.0, 1.5 and 2.0 mg/l) for embryogenic callus induction. Each treatment was repeated twice.

The explants were maintained for 8 weeks in the culture room supplied with standard photoperiod and temperature i.e. under 16 hours photoperiod at illumination of 1 000 Lux and the temperature was maintained at 25 ± 1 ºC. The morphogenetic responses were observed 8 weeks after the initiation of the cultures and the percentage of callus were scored after 6 weeks. The optimum resultant callus was transferred to callus development medium for somatic embryo development.

Below is the list of media with different combinations of plant growth regulators that were used in this study.

1. MS + 0.0 mg/l 2, 4-D (as control)
2. MS + 0.1 mg/l 2, 4-D
3. MS + 0.2 mg/l 2, 4-D
4. MS + 0.3 mg/l 2, 4-D

5. MS + 0.4 mg/l 2, 4-D
6. MS + 0.5 mg/l 2, 4-D
7. MS + 0.6 mg/l 2, 4-D
8. MS + 0.7 mg/l 2, 4-D
9. MS + 0.8 mg/l 2, 4-D
10. MS + 0.9 mg/l 2, 4-D
11. MS + 1.0 mg/l 2, 4-D
12. MS + 1.2 mg/l 2, 4-D
13. MS + 1.4 mg/l 2, 4-D
14. MS + 1.6 mg/l 2, 4-D
15. MS + 1.8 mg/l 2, 4-D
16. MS + 2.0 mg/l 2, 4-D
17. MS + 2.2 mg/l 2, 4-D
18. MS + 2.4 mg/l 2, 4-D
19. MS + 2.6 mg/l 2, 4-D
20. MS + 2.8 mg/l 2, 4-D
21. MS + 3.0 mg/l 2, 4-D
22. MS + 0.1 mg/l BAP + 0.0 mg/l 2, 4-D
23. MS + 0.5 mg/l BAP + 0.0 mg/l 2, 4-D
24. MS + 1.0 mg/l BAP + 0.0 mg/l 2, 4-D
25. MS + 1.5 mg/l BAP + 0.0 mg/l 2, 4-D
26. MS + 2.0 mg/l BAP + 0.0 mg/l 2, 4-D
27. MS + 0.1 mg/l BAP + 0.1 mg/l 2, 4-D
28. MS + 0.5 mg/l BAP + 0.1 mg/l 2, 4-D
29. MS + 1.0 mg/l BAP + 0.1 mg/l 2, 4-D

30. MS + 1.5 mg/l BAP + 0.1 mg/l 2, 4-D
31. MS + 2.0 mg/l BAP + 0.1 mg/l 2, 4-D
32. MS + 0.1 mg/l BAP + 0.5 mg/l 2, 4-D
33. MS + 0.5 mg/l BAP + 0.5 mg/l 2, 4-D
34. MS + 1.0 mg/l BAP + 0.5 mg/l 2, 4-D
35. MS + 1.5 mg/l BAP + 0.5 mg/l 2, 4-D
36. MS + 2.0 mg/l BAP + 0.5 mg/l 2, 4-D
37. MS + 0.1 mg/l BAP + 1.0 mg/l 2, 4-D
38. MS + 0.5 mg/l BAP + 1.0 mg/l 2, 4-D
39. MS + 1.0 mg/l BAP + 1.0 mg/l 2, 4-D
40. MS + 1.5 mg/l BAP + 1.0 mg/l 2, 4-D
41. MS + 2.0 mg/l BAP + 1.0 mg/l 2, 4-D
42. MS + 0.1 mg/l BAP + 1.5 mg/l 2, 4-D
43. MS + 0.5 mg/l BAP + 1.5 mg/l 2, 4-D
44. MS + 1.0 mg/l BAP + 1.5 mg/l 2, 4-D
45. MS + 1.5 mg/l BAP + 1.5 mg/l 2, 4-D
46. MS + 2.0 mg/l BAP + 1.5 mg/l 2, 4-D
47. MS + 0.1 mg/l BAP + 2.0 mg/l 2, 4-D
48. MS + 0.5 mg/l BAP + 2.0 mg/l 2, 4-D
49. MS + 1.0 mg/l BAP + 2.0 mg/l 2, 4-D
50. MS + 1.5 mg/l BAP + 2.0 mg/l 2, 4-D
51. MS + 2.0 mg/l BAP + 2.0 mg/l 2, 4-D

3.2.3 Double Staining Method for Identification of Embryogenic Callus

Double staining is a two-step staining process using two stains i.e., acetocarmine and Evan's blue. This technique has been used for staining cells and used to distinguish embryogenic cells from non-embryogenic cells with the addition of 2.0% (w/v) acetocarmine to the callus first, followed by 0.5% (w/v) Evan's Blue.

3.2.3.1 Preparation of 2.0% Acetocarmine

Acetocarmine was prepared by mixing 45 ml of glacial acetic acid with 55 ml of H_2O. Then, 2.0g of carmine were added into a beaker containing 45% glacial acetic acid solution using a pipette. The beaker was placed on a stir plate in fume hood. The solution was stirred and boiled gently for 5 minutes on highest setting temperature using hot plate. After 5 minutes, the solution was filtered using Whatman filter paper and stored at room temperature.

3.2.3.2 Preparation of 0.5% Evan's Blue

Evan's Blue stain was prepared by adding 0.5g of Evan's Blue to 100 ml of H_2O in 250-ml flask. The solution was mixed together by swirling them gently.

3.2.3.3 Double Staining Procedure

In order to distinguish between embryogenic and non-embryogenic callus, small pieces of callus (2.0-5.0 mm in size) was placed on a glass slide and a few drops of 2.0% (w/v) acetocarmine were dropped to the callus until it submerged. The callus was then gently divided into small pieces in the acetocarmine using forceps and heated over a low flame for a few seconds. The acetocarmine stained cells were washed 2-3 times with water to remove all liquid, then a few drops of 0.5% (w/v) Evan's Blue was applied to the callus. After 30 seconds, the double stained cells were washed 2-3 times with water. After removing all the water, 1-2 drops of glycerol were added to the stained cells to prevent drying of the stained cells and observed under microscope using illumination from beneath the specimen.

3.2.4 Measurement of The Percentage of Callus Formation

In order to study callus formation in different hormonal combinations from leaf and petiole explants, direct observations were done based on the quantity and quality of the callus. The quality of the callus involved various factors such as the colour of the callus, compression and structure of the callus, whereas, the quantity of callus was focused on the percentage of callus per explant area. The percentage of callus per explant was obtained by dividing the area of explants that produced callus with the total area of original explants.

Percentage of callus induction:

$$\frac{\textit{Area of explants that produced callus}}{\textit{Total area of explants used}} \times 100\%$$

3.2.5 Statistical Analysis

All data and variables were statistically analyzed using SPSS statistical package version 11. Values are presented as mean ± SE. Mean percentage and Multiple Range Analysis were done on all data, using 95% LSD intervals method.

3.3 RESULTS

In order to induce callus, two different types of explants derived from *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red* were selected for this study i.e. leaf and petiole explants. The results showed that callus could be induced from both leaf and petiole explants. Callus tissues were basically initiated from the cut ends and the epidermal surfaces of the explants. The initiation of callus normally started 2-3 weeks after inoculation and subsequently 4-6 weeks after culture establishment, callus was fully induced on the entire surfaces of the leaf and petiole explants (Plate 3.1A and 3.1B). The percentage of callus obtained from leaf and petiole explants were also determined. The leaf explants produced significantly more callus than petioles (Table 3.1, Fig. 3.1).

The results revealed that the amount of callus obtained depended on different types of explants used. Two different colours of callus were obtained from the experiments i.e. yellowish and green callus, which is compact and nodular in structures. Most of the callus induced at 0.1-1.0 mg/l 2,4-D was compact, yellowish and nodular structure. The callus initially grew well but after 7 weeks, the growth and development of the callus were reduced. Apart from that, increasing 2,4-D concentration (1.2-2.0 mg/l) was associated with a progressive reduction in callus induction, reduced the percentage of greenish callus and increased the percentage of yellowish compact and nodular callus.

The mean percentage of callus was not significantly different when different concentrations of BAP and 2,4-D were used. The use of 2,4-D in combination with 1.0 mg/l BAP resulted in greater amounts of callus from both explants. The results also showed that the growth rate of callus was significantly influenced by different culture conditions such as concentrations of plant growth regulators and different types of media that are favorable for callus growth. The callus derived from media supplemented with combinations of BAP and NAA was green, compact and nodular in structures.

The callus obtained from this experiment was further identified using double staining method. Embryogenic callus were identified whereby the staining exhibited positive results using aceto-carmine and Evans Blue. The results revealed that the green nodular callus was embryogenic whereas yellowish nodular callus was not embryogenic (Plate 3.1A and 3.1B).

Based on the present investigation, the highest amount of embryogenic and regenerative callus was obtained from leaf explant of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red* cultured on MS media supplemented with 1.0mg/l BAP, 0.1-0.5mg/l 2,4-D, 3.0% (w/v) sucrose and solidified with 0.2% (w/v) phytagel. After 2 months of incubation in the somatic embryogenic medium, the embryogenic callus was successfully developed into *in vitro* plantlets after being transferred into regeneration or development medium for another 2 months. The non-embryogenic callus could not develop into *in vitro* plantlets

and could only produce root-like structures after being subcultured onto regeneration or development media.

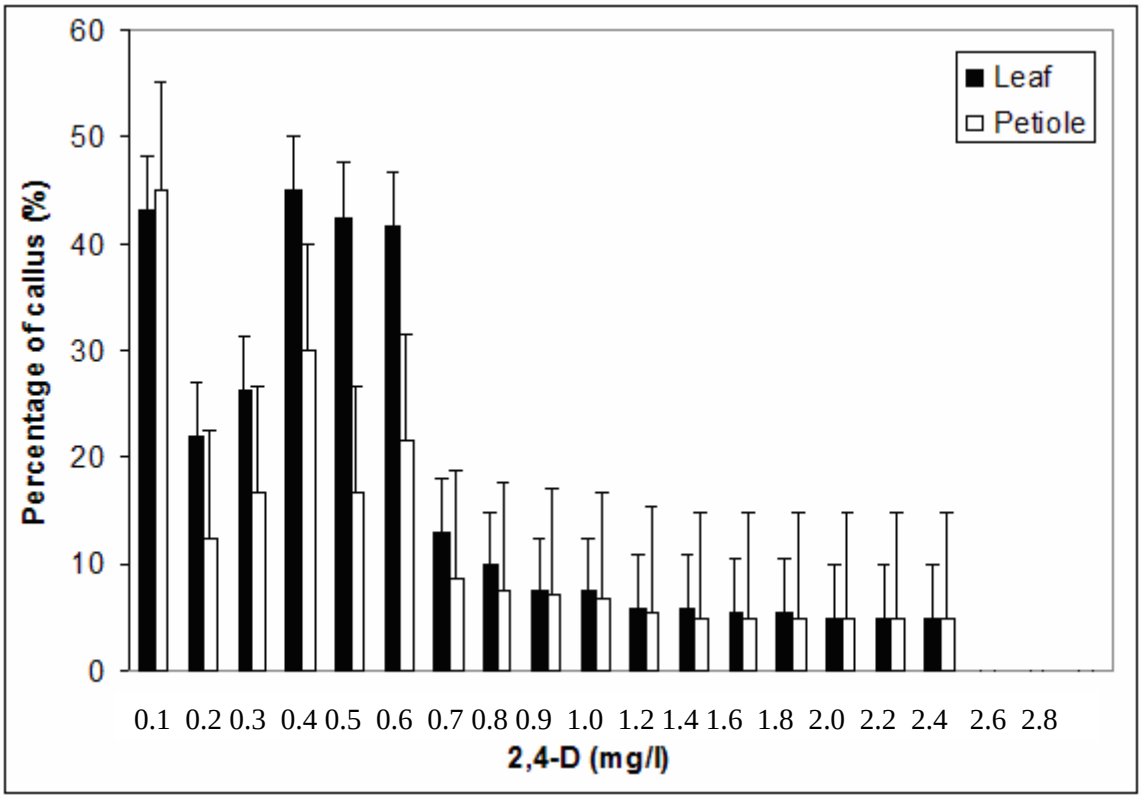


Fig. 3.1: The mean percentage of callus obtained from leaf and petiole explants of *Begonia* x *hiemalis* Fotsch. with different concentrations of 2,4-D. The cultures were kept at 25 ± 1 ºC with 16 hours light and 8 hours dark. Data were obtained after 8 weeks of inoculation.

Table 3.1: Mean percentage of callus obtained from leaf and petiole explants of *Begonia* x *hiemalis* Fotsch. cultured on MS supplemented with different concentrations of BAP and 2,4-D. The cultures were maintained at 25 ± 1 ºC with 16 hours light and 8 hours dark. Data were obtained after 8 weeks of inoculation.

Combination of BAP and 2,4-D (mg/l)		Percentage of callus (%)			
BAP	**2,4-D**	**Leaf explant**	**Callus colour**	**Petiole explant**	**Callus colour**
0.1		$55.83\pm3.12_{d}$		$44.16\pm1.49_{b}$	
0.5		$57.50\pm3.05_{d}$		$25.00\pm1.95_{d}$	
1.0	0.1	$77.50\pm2.50_{b}$	Yellowish, green	$25.00\pm1.51_{d}$	Yellowish, green
1.5		$28.33\pm1.12_{e}$		$15.00\pm1.51_{e}$	
2.0		$23.33\pm1.42_{e}$		$7.92\pm0.74_{f}$	
0.1		$80.00\pm2.13_{b}$		$27.50\pm3.29_{d}$	
0.5		$91.25\pm2.23_{a}$		$61.67\pm6.49_{d}$	
1.0	0.5	$80.00\pm0.00_{b}$	Yellowish, green	$38.33\pm6.13_{b}$	Yellowish
1.5		$70.00\pm2.13_{c}$		$33.33\pm3.96_{c}$	
2.0		$91.25\pm2.62_{a}$		$13.33\pm1.42_{e}$	
0.1		$87.50\pm4.46_{a}$		$47.92\pm6.26_{b}$	
0.5		$87.50\pm4.27_{a}$		$35.83\pm5.83_{c}$	
1.0	1.0	$65.00\pm7.33_{c}$	Yellowish	$45.00\pm3.37_{b}$	Yellowish
1.5		$52.50\pm3.51_{d}$		$25.00\pm5.11_{d}$	
2.0		$80.00\pm5.77_{b}$		$24.58\pm4.32_{d}$	

Table 3.1: Continued.

Combination of BAP and 2,4-D (mg/l)		Percentage of callus (%)			
BAP	**2,4-D**	**Leaf explant**	**Callus color**	**Petiole explant**	**Callus color**
0.1		$80.00\pm4.44_{b}$		$15.83\pm1.93_{e}$	
0.5		$70.00\pm4.61_{c}$		$12.50\pm1.69_{e}$	
1.0	1.5	$80.00\pm4.92_{b}$	Yellowish	$7.92\pm1.30_{f}$	Yellowish
1.5		$55.00\pm4.69_{d}$		$8.33\pm2.33_{f}$	
2.0		$79.16\pm5.70_{b}$		$5.42\pm0.42_{f}$	
0.1		$85.83\pm2.28_{a}$		$7.08\pm1.30_{f}$	
0.5		$48.33\pm7.47_{d}$		$7.50\pm1.69_{f}$	
1.0	2.0	$69.16\pm6.33_{c}$	Yellowish	$5.83\pm0.56_{f}$	Yellowish
1.5		$83.75\pm6.72_{b}$		$37.50\pm2.5_{f}$	
2.0		$71.66\pm5.48_{c}$		$7.50\pm1.69_{f}$	

* Figure followed by the same letter in the columns are not significantly different at $P<0.05$.

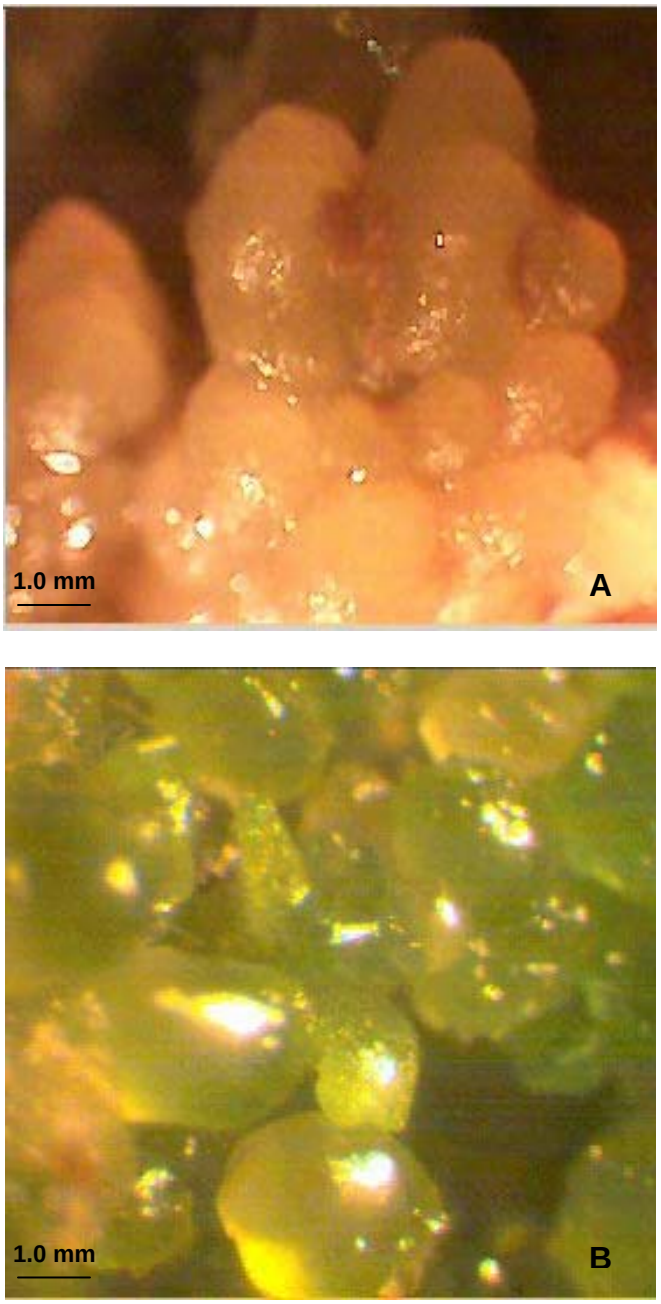


Plate 3.1: (A) Yellowish and (B) greenish callus from leaf explant of *Begonia* x *hiemalis* Fotsch. on MS medium supplemented with 1.0 mg/l BAP and 0.1 mg/l 2,4-D after 6 weeks incubation. The cultures were grown in culture room with temperature regulated at 25±1ºC and 16 hours of illumination in 24 hours cycle.

3.4 SUMMARY

1. The highest amount of embryogenic callus was obtained from leaf explants cultured on MS medium containing 3.0% (w/v) sucrose, 0.2% (w/v) phytagel and supplemented with 1.0mg/l BAP and 0.1-0.5mg/l 2, 4-D.

2. Most of the callus obtained in this experiment was nodular and compact in structures.

3. Two different colours of callus were obtained during callus induction i.e., yellowish which was not embryogenic and green callus which was embryogenic.

4. The embryogenic callus was successfully developed into *in vitro* plantlets after being transferred into regeneration or development medium, whereas non-embryogenic callus only produced root-like structures and did not develop further.

CHAPTER 4

SOMATIC EMBRYOGENESIS IN *Begonia* x *hiemalis* Fotsch.

4.1 EXPERIMENTAL AIMS

Plant regeneration from *in vitro* cultures can be obtained by embryogenesis and organogenesis (Filho and Hattori, 1997). Regeneration in *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red* was accomplished through organogenesis. Apart from organogenesis, somatic embryogenesis which significantly reflected embryo manipulation, were also carried out in this study. The first stage of somatic embryos is known as embryogenic cells (predetermined cells), which are small in size, dense cytoplasm and dividing rapidly. From embryogenic cells, their competence to develop into somatic embryos is similarly like in zygotic embryos. They are bipolar and bear typical embryogenic organs, the radical, hypocotyls and cotyledons (Von Arnold *et al*., 2002).

Plant cells are totipotent, i.e. having the ability to regenerate into complete plant from single cell. The process of somatic embryo induction involved various factors including genetic background, different types of hormones and explants, sucrose concentration, agar and environmental factors such as light, temperature etc. Amongst these factors, hormone combinations always become the main factor for early induction of somatic embryogenesis. Results from cell cultures studies indicated that this process is elicited by growth regulators, especially auxin and cytokinin (Liu *et al*., 1993). Previous research also revealed that 2,4-D was favoured for the induction of somatic embryos in other species

(Moghaddam and Taha, 2005; Devi *et al*., 2004; Jalil *et al*., 2003; Pareek and Kothari, 2003).

The incorporation of liquid-based medium for somatic embryo induction could produce plants more efficiently than the conventional tissue culture procedures. Direct regeneration through conventional tissue culture method could only produce low numbers of cloned plants whereas suspension cultures could produce more plants. Suspension cultures are normally initiated by transferring pieces of undifferentiated tissues or callus to a liquid medium, which is agitated during incubation (Street, 1977). Movement of the liquid medium facilitates fragmentation of tissues leading to smaller units and helps in their gaseous exchange (Narayanaswamy, 1977). During the period of culture, there is a peak in mitosis at about seven days after culture followed by a gradual fall in frequency after two weeks culminating in a complete cessation of mitosis in three weeks (Torrey *et al.*, 1962).

Thus, the aim of this chapter was to obtain the optimum medium for the induction of direct somatic embryos using *in vitro* plantlets derived from two different treatments, which is labeled as Treatment 1 and Treatment 2. Two different types of explants were selected in this experiment. Previous chapter (Chapter 2) showed that leaf and petiole explants were the most responsive explants for the induction of micro shoots through organogenesis process. Thus both explants were used in this study. Leaf and petiole explants of four-month-old plantlets obtained from *in vitro* regeneration protocol were selected as explant sources. In order to find out the best medium for somatic embryogenesis, various media

with different concentrations of growth regulators were selected in this study. The explants were cultured onto MS media supplemented with different concentrations of growth regulators including 2,4-D, BAP in combination with 2,4-D and also TDZ.

Apart from growth regulators, the influence of explant origin, casein hydrolysate, L-Proline, sucrose, 2,3,5-triioobenzoic acid (TIBA-auxin polar transport inhibitor), 4-amino 3,5,6-trichloropiconilic acid (Picloram), coconut milk and photoperiod were also investigated. Picloram is known as herbicide which has auxin action similar to 2,4-D (Kafford and Caso, 1966).

As a result, a new protocol was developed for production, development, maintenance and regeneration of direct somatic embryos on solid and liquid media cultures. The protocol derived from this experiment would be developed to produce suspension cultures of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red.* The somatic embryos obtained in this experiment would also be used for synthetic seeds production in Chapter 5. Finally, microscopic studies of different stages of somatic embryos were also examined using scanning-electron microscope.

4.2 MATERIALS AND METHODS

4.2.1 Plant Materials and Culture Conditions

Petioles obtained from intact plants (4-month-old) of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red* were cultured on MS medium supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA, 3.0% (w/v) sucrose and 0.8% (w/v) technical agar. The petioles were washed with tap water for ½ to 1 hour. Under aseptic conditions, petiole segments were surface sterilized using 50.0% (v/v) sodium hypochlorite solution for 1 minute. The petioles were then washed three times with sterile distilled water and followed by 70.0% (v/v) alcohol for 1 minute, then, they were washed again three times with sterile distilled water.

The petiole segments were aseptically cut (5.0 mm in length) and grown onto solid medium, which consisted of MS medium supplemented with 1.0 mg/l BAP, 1.0 mg/l NAA, 3.0% (w/v) sucrose and gelled with 0.8% (w/v) technical agar. The pH of the medium was adjusted to 5.8 prior to sterilization in an autoclave at 121 ºC and 105 kPa for 20 minutes. Cultures were incubated in a controlled environment at 25 ± 1 ºC with a 16-hour photoperiod and irradiance of 1 000 Lux, supplied by Cool White fluorescent lamps.

Shoot and root formation occurred only when 1.0 mg/l BAP and 1.0 mg/l NAA were added simultaneously to the medium. The cultures were incubated for 8 weeks to enhance micro shoot elongation.

4.2.2 Screening for Suitable Age and Different Types of Explants for Direct Somatic Embryogenesis

Two-month-old micro shoots were subcultured onto MS medium supplemented with 0.2% charcoal, 1.0 mg/l $GA_{3,}$ 3.0% (w/v) sucrose and solidified with 0.8% (w/v) technical agar. This is known as Treatment 1. After 2-6 months of incubation with the plant height of 4-5 cm, the leaves and petioles were chosen as explants from *in vitro* grown plantlets. For the purpose of somatic embryos induction, two different explants such as leaves and petioles were used derived from the *in vitro* plantlets. It is known that the best explants, which could produce optimum growth, are the second and the third leaf of the plant. The leaf explants were cut approx. 0.5 cm x 0.5 cm whereas the petiole explants were 0.5 cm in length, and then they were cultured onto the selected solid media. The explants were cultured onto MS medium supplemented with 3.0% (w/v) sucrose and 0.8% (w/v) technical agar. The cultures were incubated at 25 ± 1ºC with a 16-hour photoperiod and irradiance of 1 000 Lux, supplied by Cool White fluorescent lamps.

Apart from studying the effect of different ages of *in vitro* plantlets, the effects of young leaves obtained from *in vitro* stock plants were also studied. Thus, different positions of leaves starting from second leaves to the sixth leaves were cultured onto optimum media for somatic embryo induction.

4.2.3 Screening for Suitable Growth Regulators for Direct Somatic Embryogenesis

Different concentrations of TDZ (0.05-2.00 mg/l) were used to obtain somatic embryogenesis in Begonia. Apart from that, different combinations of other growth regulators and additives including TDZ, 2,4-D, BAP, kinetin, 2-iP, casein hydrolysate, coconut milk, L-Proline, Picloram, and TIBA with several combinations were tested for; a) inducing proliferation of embryogenic callus b) inducing and converting proembryos into cotyledonary embryos. By using MS media supplemented with different types of growth regulators and additives, the optimum somatic embryo induction medium (SEIM) was identified in this study.

The following are the list or media with different combinations of plant growth regulators that were used in this study.

1. MS + 0.05 mg/l TDZ
2. MS + 0.1 mg/l TDZ
3. MS + 0.2 mg/l TDZ
4. MS + 0.4 mg/l TDZ
5. MS + 0.5 mg/l TDZ
6. MS + 0.6 mg/l TDZ
7. MS + 0.8 mg/l TDZ
8. MS + 1.0 mg/l TDZ
9. MS + 1.2 mg/l TDZ
10. MS + 1.4 mg/l TDZ
11. MS + 1.6 mg/l TDZ

12. MS + 1.8 mg/l TDZ
13. MS + 2.0 mg/l TDZ
14. MS + 0.5 mg/l 2,4-D
15. MS + 0.5 mg/l kinetin
16. MS + 0.5 mg/l 2iP
17. MS + 0.25 mg/l BAP + 0.125 mg/l 2,4-D
18. MS + 0.5 mg/l BAP + 0.125 mg/l 2,4-D
19. MS + 0.5 mg/l BAP + 0.25 mg/l 2,4-D
20. MS + 0.5 mg/l kinetin + 0.25 mg/l 2,4-D
21. MS + 0.5 mg/ zeatin + 0.25 mg/l 2,4-D
22. MS + 0.5 mg/l 2iP + 0.25 mg/l 2,4-D
23. MS + 0.5 mg/ kinetin + 0.25 mg/l 2,4-D + 20 ml/l coconut milk
24. MS + 0.5 mg/l 2iP + 0.25 mg/l 2,4-D + 20 ml/l coconut milk
25. MS + 1.0 mg/l BAP + 0.1 mg/l 2,4-D (as control)
26. MS + 1.0 mg/l kinetin + 0.1 mg/l 2, 4-D
27. MS + 1.0 mg/l BAP + 0.1 mg/l 2, 4-D + 0.5 mg/l Picloram
28. MS + 1.0 mg/l BAP + 0.1 mg/l 2, 4-D + 0.5g/l L-Proline
29. MS + 1.0 mg/l BAP + 0.1 mg/l 2, 4-D + 0.5g/l L-Proline + 1.0 mg/l TIBA
30. 1/2MS + 1.0 mg/l BAP + 0.1 mg/l 2, 4-D + 0.5g/l casein hydrolysate

Subsequently, the explants or callus formed were examined macroscopically and microscopically for the presence and formation of embryogenic bodies e.g., globular, heart-shaped, torpedo-shaped, cotyledonary-stage and formation of plantlets, at the different time interval, e.g. 7 and 14 days.

4.2.4 The Effect of Casein Hydrolysate, Sucrose, L-Proline and TIBA on Somatic Embryogenesis

MS media supplemented with 1.0 mg/l BAP, 0.1 mg/l 2,4-D, 3.0% (w/v) sucrose, 0.2% (w/v) gelrite and different types of additives such as casein hydrolysate (0, 100, 200, 300, 400 and 500 mg/l), sucrose (0, 1.0%, 2.0%, 3.0%, 4.0% and 5.0%), L-Proline (0, 0.1, 0.5, 1.0, 1.5 and 2.0 mg/l) and TIBA (0, 1.0, 2.0, 3.0 and 4.0 mg/l) were used to obtain optimum somatic embryogenesis in Begonia. By using various concentrations of the above additives added to the MS media supplemented with 1.0 mg/l BAP and 0.1 mg/l 2,4-D, the optimum somatic embryo induction medium (SEIM) was identified in this study.

4.2.5 The Effect of Explants Derived from Different Types of *In Vitro* Plantlets on Somatic Embryogenesis

In vitro plantlets obtained on MS media supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA, 3.0% (w/v) sucrose and 0.8% (w/v) technical agar were used as donor plants. Two-month-old micro shoots from the *in vitro* plantlets were subcultured on MS supplemented with 0.2% (w/v) charcoal, 1.0 mg/l GA_3, 3.0% (w/v) sucrose and solidified with 0.8% (w/v) technical agar (Treatment 1). After 2-3 months incubation when the plantlets gained 4-5 cm height, the leaf and petiole explants were chosen as explants. For Treatment 2, two-month-old micro shoots were also subcultured on MS supplemented with 0.2% (w/v) charcoal, 1.0 mg/l TIBA, 0.5 mg/l GA_3, 3.0% (w/v) sucrose and 0.8% (w/v) technical agar. After 2-3 months incubation when the height of the plantlets reached 2.5-3.5 cm, the leaf and petiole were chosen as explants.

4.2.6 The Effect of Different Photoperiod on Somatic Embryogenesis

Different photoperiods were used to study the effect of light treatment on somatic embryogenesis. In Treatment 2, the explants were cultured on MS media supplemented with 1.0 mg/l BAP, 0.1 mg/l 2,4-D, 500 mg/l casein hydrolysate, 3.0% (w/v) sucrose, 0.2% (w/v) gelrite and incubated under a 16-hours photoperiod and 24-hours darkness at 25 ± 1ºC. The explants which were treated with 16-hours photoperiod were incubated in the culture room whereas for the dark treatment (24 hours darkness), the explants were incubated for 8 weeks in the growth chamber and the temperature was set at 25 ± 1ºC and the observations were made every week.

4.2.7 Suspension Cultures of Somatic Embryos

Suspension culture protocol for Begonia was developed in this study by using the embryogenic callus grown on callus induction medium (CIM). Embryogenic callus clumps (0.5g) derived from Treatment 2 were cut into small pieces and then transferred into 125-ml conical flasks containing 20.0 ml of MS liquid medium supplemented with 1.0 mg/l BAP in combinations with 0.1 mg/l 2,4-D, 0.5 g/l L-Proline and 3.0% (w/v) sucrose (Suspension culture medium or SCM) at 25 ± 1°C after 6 weeks of culture in callus induction medium. After 1 week of incubation, the suspension cultures were allowed to settle and 10.0 ml of supernatant were removed from the conical flasks and replaced by 10.0 ml of fresh medium. The experiment was repeated for 3-6 weeks to dissociate the callus into single cells and small cell clumps. The suspension cultures were maintained on

a horizontal shaker (100 rpm) in the culture room at 25 ± 1°C under a 16 hours photoperiod.

After 6 weeks being cultured in the SCM, the callus was transferred into embryo development medium (EDM) i.e. MS supplemented with 1.0 mg/l BAP, 1.0 mg/l NAA, 3.0% (w/v) sucrose and 0.2% (w/v) gelrite for another 4 weeks. Different stages of somatic embryos were identified and after 4 weeks incubation, the embryoids were then subcultured into optimum regeneration medium, which were determined before (Chapter 2) (MS supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA). Each experiment contained 6 replicates and the experiment was repeated twice.

4.2.8 Micromorphological Studies of Different Stages of Somatic Embryos

The specimens were dried up prior to run the specimen in the vacuum system. Somatic embryos at different stages of development was used to study morphological event that takes place in the process of somatic embryogenesis. The samples were treated with several steps including air drying technique, low temperature technique and chemical bonding technique followed by critical point drying technique.

Air-drying technique was done in the normal room whereby the selected samples were placed in the room temperature. The specimen was washed with alcohol and sticked on

using liquid carbon. This was done simultaneously in order to avoid the carbon ink from drying.

Selected samples were treated with different types of chemicals. The sample was incubated in 30 ml of gluteraldehyde mixed with 30 ml of phosphate buffer for 1 hour at room temperature. Then, the sample was rinsed with phosphate buffer solution and distilled water in 1:1 mixture. The sample was incubated in Osmium (4%) and distilled water in 1:1 mixture for 14 hours at 4º C. The sample was rinsed with distilled water and fixed using the ethyl alcohol series (10% to 100%) for 15 min. each step, and followed by 3:1, 1:1 and 1:3 ethyl alcohol and acetone for 20 min. Then, the sample was incubated in Aseton (100%) for 20 min and this step was repeated four times. Lastly, the sample was sticked onto the specimen holder, plated and double-checked for conformation.

The sample was dried up in the liquid CO_2 for several times using CPD equipment to replace the acetone with carbon dioxide. The temperature was increased until liquid CO_2 changed to gas to avoid surface tension of the sample. Then, the specimen was ready to be plated with gold dust to stabilize the term and electricity of the sample. The sample was attached to special aluminum pin using conductive carbon cement. Then the pin was placed inside of SPI-Module Sputter Coaster chamber. The air of the chamber was placed with argon gas using a pump, which has been attached to the gold coater equipment. The sample was coated with gold particle for 60 seconds. Finally the specimen was observed using SEM (JEOL, JSM. 6400, Tokyo, Japan).

4.2.9 Statistical Analysis

All data and variables were statistically analyzed using SPSS statistical package version 11. Values are presented as mean ± SE. One-way ANOVA and Multiple Range Analysis were done on all data, using 95% LSD intervals method.

4.3 RESULTS

4.3.1 The Effect of Different Ages and Different Types of Explants on Somatic Embryogenesis

Prior to somatic embryo induction, the different ages of the plantlets obtained from previous experiments ranging from 2–6-month-old were used to study the effect of plantlets' age on *in vitro* response. The plantlets which were subcultured onto development media were selected as donor plants. Figure 4.1 shows the effect of different ages of the plantlets on organogenesis. Based on this experiment, it was found that increasing the age of plantlets caused the decrease in organogenesis abilities for leaf and petiole explants. There was no significant difference between the mean percentages of shoot formation between two-month-old plantlets with four-month-old, either for leaf and petiole explants. However, the data showed that there was significant difference between the percentages of shoot formation in three, five and six months old explants. The results obtained also showed that two-month-old explants (both leaf and petiole) had significantly higher mean score than the other ages. Thus, two-month-old plantlets were chosen for the purpose of callus and somatic embryo induction in this chapter.

Direct somatic embryo induction was also attempted using two different explants i.e. leaves and petioles. Figure 4.2 showed the effect of different positions of explants starting from the second to the sixth position of leaf on organogenesis.

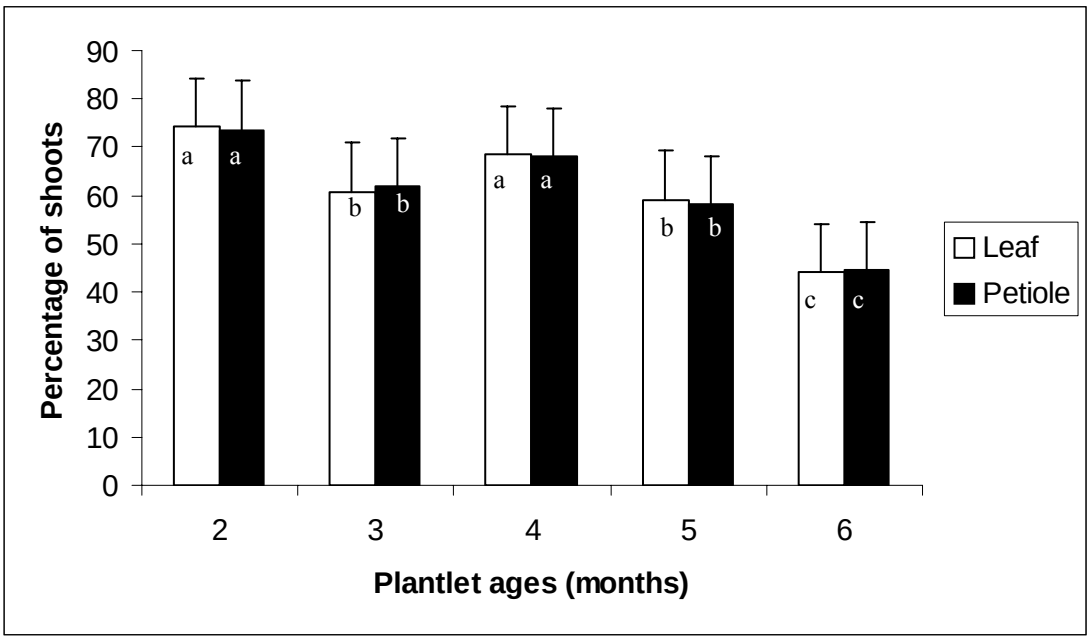


Figure 4.1: The shoot formation from the explants taken from plantlets at different ages (months) of plantlets.

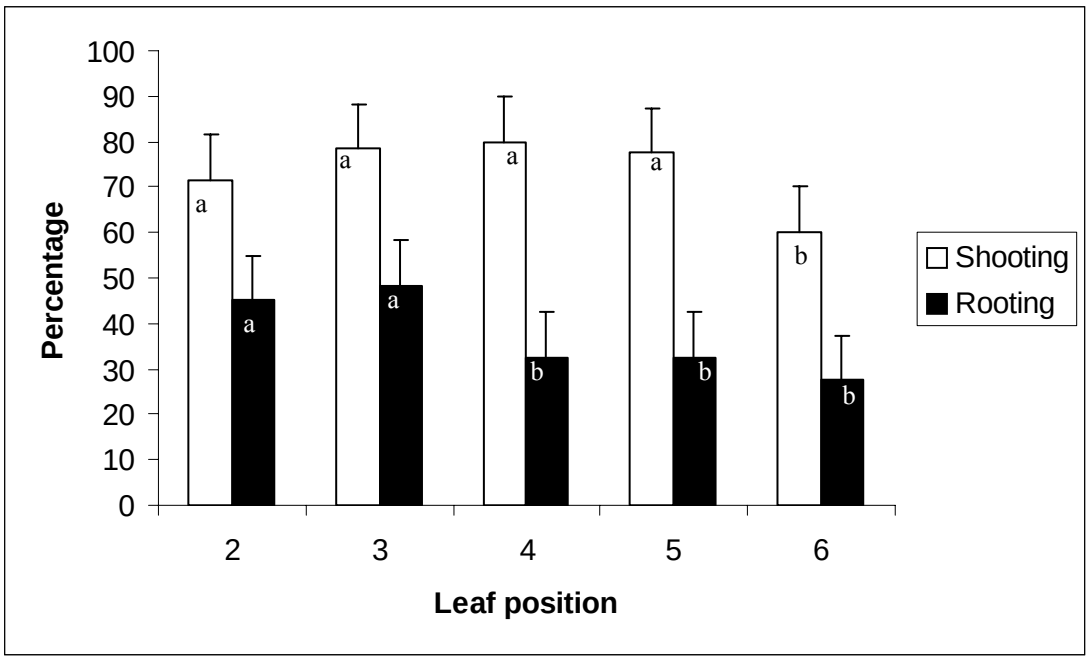


Figure 4.2: The mean percentage of shoots and roots formed from different positions of the leaf explants.

The results showed that the second, third, forth and fifth position of leaf gave optimum responses on shooting and rooting process (Figure 4.2). The observations revealed that the sixth leaves just exhibited moderate response in shooting and rooting as compared to other leaves. Hence, from this experiment it was observed that second and third leaves were selected for somatic embryo induction.

4.3.2 The Effect of Different Concentrations of Plant Growth Regulators on Somatic Embryogenesis

To study the effect of plant growth regulators on somatic embryo induction, 2,4-D and TDZ utilized. The results revealed that, 2, 4-D (0-3.0 mg/l) and TDZ (0-2.0 mg/l) applied singly could not produce direct somatic embryogenesis from leaf and petiole explants. The callus obtained was observed macroscopically. Yellowish and compact callus were obtained from both explants either in MS medium supplemented with 2,4-D (Table 4.1) or TDZ alone (Table 4.2). The explants grew bigger after 1 week being cultured on callus induction medium. The explants were then covered by proliferation of callus. However, callus turned brown and did not develop into shoots even though they were subcultured onto regeneration medium.

The mean percentage of callus obtained from leaf and petiole explants were determined (Table 4.1 and Table 4.2). The results revealed that different types of explants produced different amount of callus. The leaf explants produced significantly more callus than petiole explants on the same medium. Mean percentage of callus was not significantly

different ($p>0.05$) and after 8 weeks of culture, the yellowish callus slowly turned into brown and did not develop into shoots even though they were subcultured onto regeneration medium.

Table 4.1: Percentage of embryogenic callus from leaf and petiole explants of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red*, cultured on MS medium supplemented with different concentrations of 2,4-D. The cultures were maintained at 25 ± 1 ºC with 16 hours light and 8 hours dark. Data were obtained after 8 and 12 weeks of culture.

MS medium + 2,4-D (mg/l)	Callus induction (% ± SE)		Observations	
	Leaf*	Petiole*	(8 weeks)	(12 weeks)
0	15.00 ± 3.10^{c,d}	10.00 ± 1.05^{d}	Rooting	Rooting
0.2	63.75 ± 2.10^{a}	60.71 ± 3.27^{a}	Nodular, compact, yellowish callus	Yellowish callus browning
0.4	46.15 ± 2.16^{b}	26.49 ± 3.23^{b}	Nodular, compact, yellowish callus	Yellowish callus
0.6	44.62 ± 4.13^{b}	15.00 ± 2.20^{c,d}	Nodular, compact, yellowish callus	Yellowish callus
0.8	40.00 ± 3.34^{b}	10.00 ± 1.10^{d,e}	Friable, nodular, whitish callus	Necrotic
1.0	20 21 ± 1.10^{c}	10.00 ± 0.90^{d,e}	Friable, nodular, whitish callus	Necrotic
1.2	5.00 ± 0.00^{e}	5.00 ± 0.00^{e}	Friable, whitish callus	Necrotic
1.4	5.00 ± 0.00^{e}	5.00 ± 0.00^{e}	Necrotic	Necrotic

* Mean values within a column by the same letters are not significantly different at the 0.05 level according to LSD test between the same explant.

Table 4.1: Continued

MS medium + 2,4-D (mg/l)	Callus induction (% ± SE)		Observations	
	Leaf*	Petiole*	(8 weeks)	(12 weeks)
1.6	5.00 ± 0.00 e	10.00 ± 0.70 d,e	Friable, whitish callus	Necrotic
1.8	5.00 ± 0.00 e	5.00 ± 0.00 e	Friable, whitish callus	Necrotic
2.0	10.00 ± 0.00d	5.00 ± 0.00 e	Friable, whitish callus	Necrotic
2.2	5.00 ± 0.00e	5.00 ± 0.00 e	Friable, whitish callus	Necrotic
2.4	5.00 ± 0.00 e	5.00 ± 0.00 e	Friable, whitish callus	Necrotic
2.6	0	0	No. response	No. response
2.8	0	0	No. response	No. response
3.0	0	0	No. response	No. response

* Mean values within a column followed by the same letters are not significantly different at the 0.05 level according to LSD test between the same explant.

Table 4.2: Percentage of embryogenic callus from leaf and petiole explants of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red,* cultured on MS medium supplemented with different concentrations of TDZ. The cultures were maintained at 25 ± 1 ºC with 16 hours light and 8 hours dark. Data were obtained after 8 and 12 weeks of culture.

MS medium + TDZ (mg/l)	Callus induction (% ± SE)		Observations	
	Leaf*	Petiole*	(8 weeks)	(12 weeks)
0	15.00 ± 3.10^{g}	10.00 ± 1.05^{d,e}	Rooting	Rooting
0.05	64.00 ± 5.16^{c}	48.00 ± 1.22^{b}	Nodular, compact, yellowish callus	Yellowish callus turning brown
0.1	83.00 ± 8.23^{a}	68.00 ± 1.75^{a}	Nodular, compact, yellowish callus	Yellowish callus turning brown
0.2	21.50 ± 8.83^{g}	14.50 ± 4.38^{d}	Nodular, compact, yellowish callus	Yellowish callus
0.3	40.00 ± 0.00^{e}	16.00 ± 5.16^{d}	Nodular, compact, yellowish callus	Yellowish callus
0.4	72.00 ± 7.88^{b}	36.00 ± 9.66^{c}	Nodular, compact, yellowish callus	Yellowish callus
0.5	56.00 ± 5.16^{d}	14.00 ± 5.16^{d}	Nodular, compact, yellowish callus	Yellowish callus turning brown
0.6	70.00 ± 1.33^{b}	40.00 ± 6.67^{c}	Nodular, compact, yellowish callus	Yellowish callus turning brown
0.7	29.00 ± 8.76^{f}	11.00 ± 5.16^{d,e}	Nodular, compact, yellowish callus	Yellowish callus turning brown
0.8	6.11 ± 2.20^{h}	5.00 ± 0.00^{e}	Nodular, compact, yellowish callus	Yellowish callus turning brown

Table 4.2: Continued

MS medium + TDZ (mg/l)	Callus induction (% ± SE)		Observations	
	Leaf*	Petiole*	(8 weeks)	(12 weeks)
1.0	5.00 ± 0.00 h	5.45 ± 1.51 e	Nodular, compact, yellowish	Yellowish callus turning brown
1.2	5.00 ± 0.00 h	7.00 ± 2.58 e	Nodular, compact, yellowish	Yellowish callus turning brown
1.4	6.50 ± 2.41 h	5.00 ± 0.00 e	Nodular, compact, yellowish	Yellowish callus turning brown
1.6	6.00 ± 2.11 h	5.00 ± 0.00 e	Nodular, compact, yellowish	Yellowish callus turning brown
1.8	6.00 ± 2.11 h	5.00 ± 0.00 e	Nodular, compact, yellowish	Yellowish callus turning brown
2.0	6.50 ± 2.41 h	5.00 ± 0.00 e	Nodular, compact, yellowish	Yellowish callus turning brown

* Mean values within a column followed by the same letters are not significantly different at the 0.05 level according to LSD test between the same explant.

4.3.3 The Effect of Casein Hydrolysate, Sucrose, L-Proline and TIBA on Somatic Embryogenesis

Different combinations of growth regulators and additives were also tested to identify embryogenic callus induction in Begonia. The mean percentage of callus formation from leaf and petiole explants was presented in Table 4.3. After 8 weeks of incubation, frequencies of explants forming embryogenic callus from green callus were scored. Based on Table 4.3, the best induction of direct somatic embryogenesis of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red* was achieved on 1/2 MS medium supplemented with 1.0 mg/l BAP, 0.1 mg/l 2, 4-D, 500 mg/l casein hydrolysate, 3.0% (w/v) sucrose and solidified with 0.2% (w/v) gelrite (Plate 4.1). After 4-5 weeks of incubation, frequencies of explants forming embryogenic callus from green callus were scored. The results also showed that combinations of BAP (0.25-1.0 mg/l) and 2,4-D (0.10-0.50 mg/l) produced green embryogenic callus and yellowish compact callus from both explants. The embryogenic callus developed into torpedo-shaped after 12 weeks of incubation period.

Table 4.3: Percentage of embryogenic callus from leaf and petiole explants of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red*, cultured on MS medium supplemented with different concentrations of cytokinins, 2,4-D and additives. The cultures were maintained at 25 ± 1 ºC with 16 hours light and 8 hours dark. Data were obtained after 8 weeks of culture.

MS medium + growth regulators (mg/l)	Callus induction (% ± SE)		Observations	
	Leaf*	Petiole*	(8 weeks)	(12 weeks)
0.25 BAP + 0.125 2,4-D	26.67 ± 5.56^{f}	22.33 ± 8.21^{e}	Nodular, compact , yellowish callus	Yellowish callus turning brown
0.5 BAP + 0.125 2,4-D	44.00 ± 2.28^{d}	41.33 ± 1.17^{c}	Nodular, compact, yellowish callus	Yellowish callus
0.5 BAP + 0.25 2,4-D	78.00 ± 6.67^{b}	77.67 ± 1.16^{a}	Nodular, compact, green and yellowish callus	Torpedo green embryoids
0.5 kinetin + 0.25 2,4-D	72.00 ± 8.62^{b,c}	34.67 ± 7.42^{d}	Nodular, compact, yellowish callus	Yellowish callus turning brown
0.5 zeatin + 0.25 2,4-D	67.33 ± 4.57^{c}	66.00 ± 1.12^{b}	Nodular, compact, yellowish and brownish callus	Yellowish callus turning brown
0.5 2iP + 0.25 2,4-D	75.33 ± 5.16^{b}	75.33 ± 7.43^{a}	Nodular, compact, yellowish and brownish callus	Yellowish callus b turning brown
0.5 kinetin + 0.25 2,4-D + 20 ml/l coconut milk	73.00 ± 9.96^{b}	33.33 ± 6.17^{d}	Nodular, compact, green and yellowish callus	Yellowish callus turning brown

Table 4.3: Continued

MS medium + growth regulator (mg/l)	Callus induction (% ± SE)		Observations	
	Leaf*	Petiole*	(8 weeks)	(12 weeks)
0.5 2iP + 0.25 2,4-D + 20 ml/l coconut milk	32.67 ± 8.83^{e}	37.33 ± 5.94^{c,d}	Nodular, compact, green and yellowish callus	Torpedo green embryoids
1.0 BAP + 0.1 2,4-D	64.00 ± 11.8^{c}	60.67 ± 13.34^{b}	Nodular, compact, green callus	Torpedo green embryoids
1.0 BAP + 0.1 2,4-D + 500 mg/l L-Proline	76.00 ± 5.07^{b}	62.00 ± 8.62^{b}	Nodular, compact, green and yellowish callus, micro shoots	Torpedo green embryoids, shoots
1.0 BAP + 0.1 2,4-D + 500mg/l L-Proline + 1.0 mg/l TIBA	41.33 ± 5.12^{d}	39.33 ± 1.16^{c,d}	Nodular, compact, green and yellowish callus	Torpedo green embryoids
1/2MS + 1.0 BAP + 0.1 2,4-D + 500 mg/l casein hydrolysate	93.33 ± 6.17^{a}	44.58 ± 2.03^{c}	Nodular, compact, green and yellowish callus	Torpedo green embryoids

* Mean values within a column followed by the same letters are not significantly different at the 0.05 level according to LSD test between the same explant.

Plate 4.1: The globular (G) (A1, A2), torpedo-shaped (T) (B) and cotyledonary-like structure (C) stages of somatic embryogenesis of *Begonia* x *hiemalis* Fotsch. incubated under 16 hours light in the optimum media after 8 weeks cultured at 25 ± 1° C.

Different concentrations of casein hydrolysate, sucrose, l-Proline, and TIBA were tested to identify the best or optimum media for direct somatic embryo induction in Begonia. Several reports also have proven the use of these additives as beneficial for the formation of somatic embryos *in vitro.* For instance, addition of casein hydrolysate in the callus induction medium was found to be beneficial. Different concentrations of casein hydrolysate (Table 4.4), L-Proline (Table 4.5), sucrose (Table 4.6), and TIBA (Table 4.7) were tested to identify the best or optimum media for somatic embryo induction in Begonia.

Table 4.4 shows that different concentrations of casein hydrolysate could induce embryogenic callus. The results in Table 4.4 showed that 100 mg/l and 500 mg/l casein hydrolysate produced the best percentage of green nodular callus as compared with other treatments. Overall observations revealed that 500 mg/l casein hydrolysate could produce the highest amount of embryogenic callus from overall percentage (30.83%) of callus i.e. 23.75%, whereas 100 mg/l casein hydrolysate could only produce 24.16% from 53.08% of overall callus. The results revealed that casein hydrolysate can be used for somatic embryo induction in Begonia and 500 mg/l casein hydrolysate was found as the best concentration in this study.

Table 4.4: Percentage of embryogenic callus from leaf and petiole explants of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red*, cultured on MS medium supplemented with 1.0mg/l BAP and 0.1mg/l 2,4-D with different concentrations of casein hydrolysate. The cultures were maintained at 25 ± 1 ºC with 16 hours light and 8 hours dark. Data were obtained after 8 weeks of culture.

	Callus induction (% ± SE)		Embryogenic callus (% ± SE)	
Casein hydrolysate (mg/l)	**Leaf***	**Petiole***	**Leaf***	**Petiole***
0	$45.00\pm2.30_a$	$49.17\pm3.58_a$	$10.44\pm1.79_b$	15.00 ± 2.61^b
100	$53.08\pm4.87_a$	$40.00\pm0.00_b$	$24.16\pm3.79_a$	$20.00\pm0.00_a$
200	$40.00\pm4.60_b$	$23.33\pm1.42_c$	8.33 ± 3.66^c	$10.83\pm0.56_b$
300	$28.33\pm2.71_c$	$25.83\pm1.49_c$	1.67 ± 1.12^d	$11.67\pm0.71_b$
400	$29.16\pm3.53_c$	$29.17\pm0.83_c$	$16.67\pm3.96_b$	$11.25\pm0.65_b$
500	$30.83\pm2.88_c$	$25.00\pm1.50_c$	$23.75\pm3.20_a$	$12.27\pm0.79_b$

* Mean values within a column followed by the same letters are not significantly different at the 0.05 level according to LSD test between the same explant.

The effects of different concentrations of L-Proline on somatic embryo induction were examined using two different explants i.e. leaves and petioles (Table 4.5). MS media supplemented with 1.0 mg/l BAP, 0.1 mg/l 2,4-D, 500 mg/l casien hydrolysate, 0.2% gelrite and different concentrations of L-Proline ranging from 0.1% (w/v) to 2.0%(w/v) were used. Formation of somatic embryos were observed in both explants within 3-4 weeks.

As shown in Table 4.5, there was no significant difference between the mean percentages of embryogenic callus in petiole but there was significant difference between mean percentage in leaf explants. The data also showed that there was no significant difference between the percentages of callus formation in leaves. However, there was significant difference between the percentages of callus from petiole explants. The optimum percentage of embryogenic callus induction was leaf explants which had a significant mean of 23.75% compared to other concentrations. The results show that 1.5-2.0 mg/l L-Proline could produce the highest percentage of embryogenic callus from leaf explants, whereas petiole explants gave the similar percentage of embrogenic callus from each treatment.

Table 4.5: Percentage of embryogenic callus from leaf and petiole explants of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red*, cultured on MS medium supplemented with 1.0mg/l BAP and 0.1mg/l 2,4-D with different concentrations of L-Proline. The cultures were grown at 25 ± 1 ºC with 16 hours light and 8 hours dark. Data were obtained after 8 weeks of culture.

	Callus induction (% ± SE)		Embryogenic callus (% ± SE)	
L-Proline (mg/l)	**Leaf***	**Petiole***	**Leaf***	**Petiole***
0	$45.00\pm2.30_{c}$	$49.17\pm3.58_{b}$	$10.44\pm1.79_{b}$	15.00 ± 2.61^{a}
0.1	0	0	0	0
0.5	$74.67\pm8.34_{a}$	$76.00\pm13.42_{a}$	$8.33\pm3.66_{c}$	$10.83\pm0.56_{a}$
1.0	$63.00\pm8.41_{b}$	$9.67\pm4.81_{c}$	$1.67\pm1.12_{d}$	$11.67\pm0.71_{a}$
1.5	$77.33\pm11.63_{a}$	$5.00\pm0.00_{c}$	$23.75\pm3.20_{a}$	$12.27\pm0.79_{a}$
2.0	$65.33\pm12.46_{b}$	$6.00\pm2.07_{c}$	$23.75\pm3.20_{a}$	$12.27\pm0.79_{a}$

* Mean values within a column followed by the same letters are not significantly different at the 0.05 level according to LSD test between the same explant.

Table 4.6 shows that different concentrations of sucrose could produce embryogenic callus. The results also showed that culturing at different sucrose concentrations on MS media had no effect on callus establishment, but influenced the callus quality. Overall observation indicated that lower concentration of sucrose (1.0%) would normally reduce the response of the explants. Apparently, high sucrose level (5.0%) was more stressful for embryogenic callus, which exhibited reduced green callus and poor callus development. In MS medium supplemented with 1.0% sucrose, greenish callus could be obtained. MS with 4.0% of sucrose was the optimum for subsequent embryogenic callus survival and growth.

Table 4.7 shows the effects of different concentrations of TIBA on somatic embryo induction were examined using two different explants i.e. leaves and petioles. Generally, both explants (leaves and petioles) successfully formed embryogenic callus on MS supplemented with 1.0 mg/l BAP, 0.1 mg/l 2,4-D, 3.0% (w/v) sucrose, 0.2% (w/v) gelrite and different concentrations of TIBA used. The results showed that the percentage of somatic embryos were not significantly different in different concentrations of TIBA. Somatic embryo formation decreased when the concentration of TIBA increased (1-3 mg/l), except for 4.0 mg/l TIBA. The callus maintained green colour and MS media with 1.0 mg/l BAP and 0.1 mg/l 2,4-D supplemented with 1.0 mg/l of TIBA was an effective medium for embryogenic callus compared to other concentrations. A mean of 38.89% embryogenic callus was obtained from the cultures.

Table 4.6: Percentage of embryogenic callus from leaf and petiole explants of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red*, cultured on MS medium supplemented with 1.0mg/l BAP and 0.1mg/l 2,4-D with different concentrations of sucrose. The cultures were grown at 25 ± 1 ºC with 16 hours light and 8 hours dark. Data were obtained after 8 weeks of culture.

	Callus induction (% ± SE)		Embryogenic callus (% ± SE)	
Sucrose %(w/v)	Leaf*	Petiole*	Leaf*	Petiole*
0	$45.00\pm2.30_{a}$	$49.17\pm3.58_{a}$	$10.44\pm1.79_{c}$	$15.00\pm2.61^{b,c}$
1.0	21.88 ± 6.55^{c}	18.75 ± 11.32^{c}	17.50 ± 6.55^{b}	20.00 ± 3.65^{b}
2.0	38.12 ± 14.67^{b}	14.68 ± 9.28^{c}	23.12 ± 8.73^{b}	23.75 ± 5.00^{b}
3.0	50.00 ± 12.30^{a}	39.68 ± 12.20^{b}	35.63 ± 10.31^{a}	30.63 ± 7.50^{a}
4.0	23.12 ± 7.04^{c}	14.68 ± 7.63^{c}	18.75 ± 8.06^{b}	13.12 ± 4.79^{c}
5.0	28.00 ± 8.20^{c}	15.33 ± 9.07^{c}	23.67 ± 5.57^{b}	18.00 ± 4.14^{b}

* Mean values within a column followed by the same letters are not significantly different at the 0.05 level according to LSD test between the same explant.

Table 4.7: Percentage of embryogenic callus from leaf explants of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red*, cultured on MS medium supplemented with 1.0mg/l BAP and 0.1mg/l 2,4-D with different concentrations of TIBA. The cultures were grown at 25 ± 1 ºC with 16 hours light and 8 hours dark. Data were obtained after 8 weeks of culture.

TIBA (mg/l)	Callus induction (% ± SE)	Embryogenic callus (% ± SE)
0	$45.00 \pm 2.30_{c}$	$10.44 \pm 1.79_{c}$
1.0	$85.00 \pm 3.98_{a}$	$38.89 \pm 1.97_{a}$
2.0	$68.71 \pm 2.77_{b}$	$31.61 \pm 1.97_{a,b}$
3.0	$40.29 \pm 1.32_{c}$	$25.59 \pm 1.20_{b}$
4.0	$27.50 \pm 1.82_{d}$	$29.17 \pm 1.73_{b}$

***** Figure followed by the same letter in the columns are not significantly different at $P<0.05$.

4.3.4 The Effect of Different Types of *In Vitro* Plantlets and Different Photoperiod on Somatic Embryogenesis

The axenic plantlets development derived from Treatment 2 were reduced in their sizes (2.5-3.5 cm height) after 6-8 weeks subcultured onto MS medium supplemented with 1.0 mg/l TIBA. The roots grew with anti-gravitropisme position whereby they elongated away from the medium area (Plate 4.2).

The effects of different light treatment on the production of somatic embryogenesis were also investigated. The results showed that mean percentage of callus was significantly different when different explants without TIBA treatment were used under different light treatment (Table 4.8). Whereas, the leaf and petiole explants derived from axenic plantlets treated with 1.0 mg/l TIBA produced the highest percentage of callus i.e. 58.67% and 36.00% after being incubated in 16 hours light 8 hours dark. The results show that dark incubation with 24-hour darkness produced complete embryogenesis cycle compared with 16 hours light 8 hours dark (Plate 4.3). Plate 4.3 also shows different stages of somatic embryos were successfully obtained which formed a globular, heart-shaped, torpedo-shaped and cotyledonary-like structure from leaf explants.

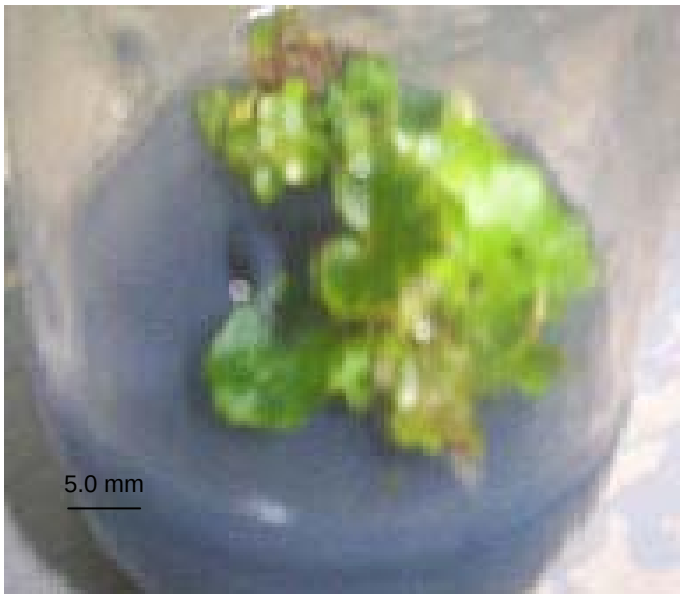


Plate 4.2: The plantlets derived from MS medium supplemented with 1.0 mg/l TIBA which were used for callus induction cultures.

Table 4.8: The effect of different treatments of axenic plantlets and light treatments on the production of embryogenic callus from leaf and petiole explants of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red* cultured on MS medium supplemented with 1.0mg/l BAP, 0.1mg/l 2,4-D and 0.5g/l casein hydrolysate. Data were obtained after 8 weeks of inoculation.

Light treatment	Observations		Embryogenic callus (% ± SE)	
	Leaf	Petiole	Leaf*	Petiole*
a) Axenic plantlets without TIBA treatment				
16hr light 8 hr dark	Green nodular callus	Green nodular callus	$10.44 \pm 1.79_{c}$	15.00 ± 2.61^{b}
24 hr dark	White nodular callus	White nodular callus	$40.44 \pm 4.68_{b}$	$40.67 \pm 5.30_{a}$
b) Axenic plantlets with TIBA treatment				
16hr light 8 hr dark	Green nodular callus	Green nodular callus	$58.67 \pm 4.68_{a}$	$36.00 \pm 1.31_{a}$
24 hr dark	White nodular callus	White nodular callus	$40.44 \pm 4.68_{b}$	$40.67 \pm 5.30_{a}$

*Figure followed by the same letter in the columns are not significantly different at $P<0.05$.

Table 4.9: The protocol for obtaining direct somatic embryogenesis of *Begonia* x *hiemalis* Fotsch. which was incubated in the dark for 24 hours.

Culture stage	MS medium with growth regulators (mg/l)					Casien hydrolysate (mg/l) or charcoal (g/l)	Culture period (weeks)	Observations
	BAP	NAA	2,4-D	TIBA	GA_3			
Plantlets induction	1.0	1.0					8	Micro shoots developed into shoots
Plantlets development				1.0			4-8	Stunted axenic plantlets with root grew upward
Embryogenic callus induction	1.0		0.1			500 mg/l	6-8	White nodular callus
Somatic embryo development					0.5		4	Development of different stages of somatic embryos
Complete plant						0.2 g	4-8	Complete plant regeneration

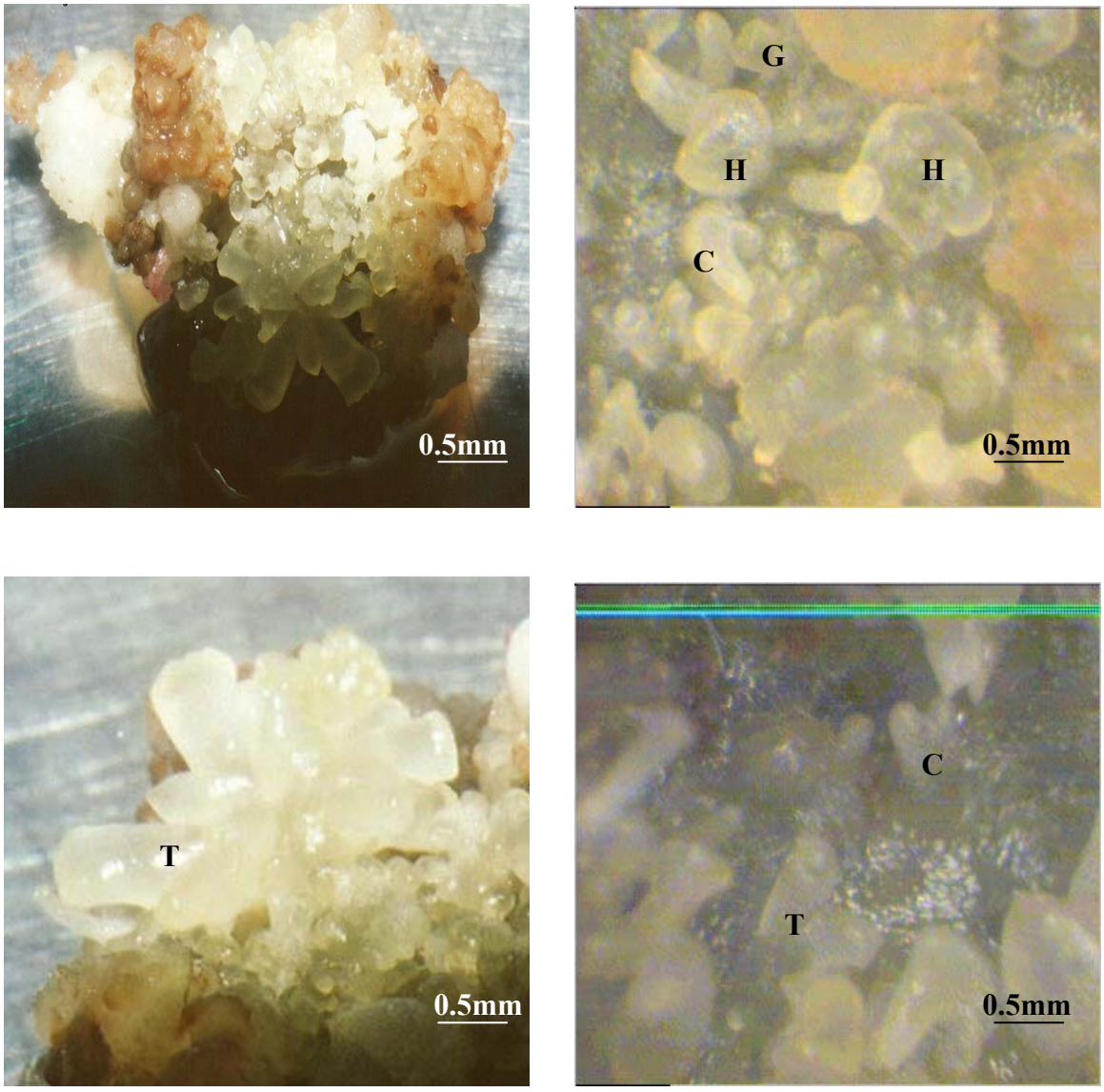


Plate 4.3: The different stages of somatic embryogenesis in leaf explant of *Begonia* x *hiemalis* Fotsch. incubated in the dark in the MS medium supplemented with 1.0mg/l BAP, 0.1mg/l 2,4-D, 500 mg/l casein hydrolysate and subcultured into 0.5 mg/l GA_3 after 12 weeks. (G-globular, H-heart-shaped, T-torpedo-shaped, C-cotyledonary-like structure of different stages of somatic embryogenesis).

4.3.5 Suspension Cultures of Somatic Embryos

The leaf and petiole explants derived from axenic plantlets treated with 1.0 mg/l TIBA were successfully cultured into CIM i.e. MS supplemented with 1.0 mg/l BAP, 0.1 mg/l 2,4-D, 3.0% (w/v) sucrose and 0.2% (w/v) gelrite. The callus were induced from both explants after being cultured for 2 weeks. The results showed that, 6 weeks was the optimum duration for callus induction. After 20-25 days of the initiation of suspension culture, globular embryos appeared on the explants or released into liquid medium (Plate 5.2). The nodular structures developed from a callus phase (Plate 4.4). Embryogenic cell suspension of Begonia developed within 2-3 weeks provided cells were transferred to a fresh medium at periodic interval. About 90.00 % of the embryoids were capable of regenerating and formed 5-15 emblings per explant (Plate 4.5).

Table 4.10: The effect of different compositions of growth media and duration of cultures per developmental stage of Begonia for suspension cultures of somatic embryos and plant regeneration.

Culture stages	MS with growth regulators (mg/l)	L-Proline (mg/l) /Charcoal (g/l)	Culture Period (weeks)	Observations
Plantlet induction	1.0 BAP + 1.0 NAA	-	8	Micro shoots
Plantlet development	1.0 TIBA	-	4-8	Retarded plantlets and anti-gravitropisme roots
Callus induction	1.0 BAP + 0.1 2,4-D		6-8	Greenish globular callus
Cell suspension	1.0 BAP + 0.1 2,4-D	500 mg L-Proline	4	Greenish callus
Embryo development	1.0 BAP + 1.0 NAA		4	Heart-shaped and torpedo-shaped embryos
Complete plant	0.5 GA_3	0.2 g Charcoals	4-8	Complete plantlet regeneration

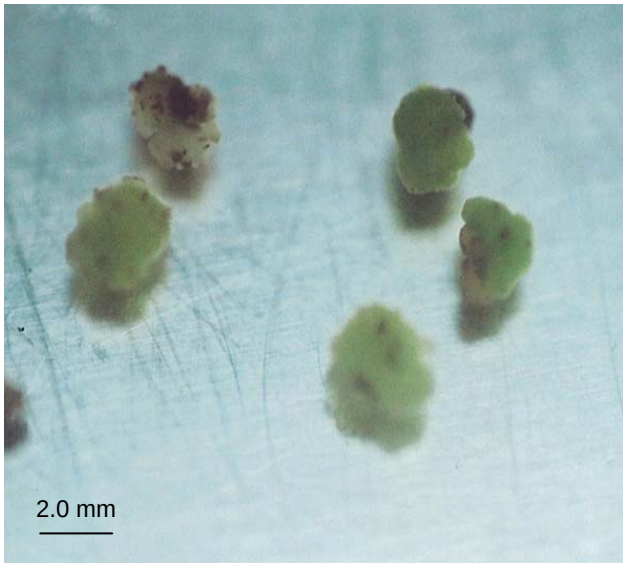


Plate 4.4: The embryoids derived from suspension cultures of *Begonia* x *hiemalis* Fotsch. cultured in MS liquid medium supplemented with 1.0 mg/l BAP in combinations with 0.1 mg/l 2,4-D, 0.5 g/l L-Proline and 3.0% sucrose after 6 weeks of incubation period.

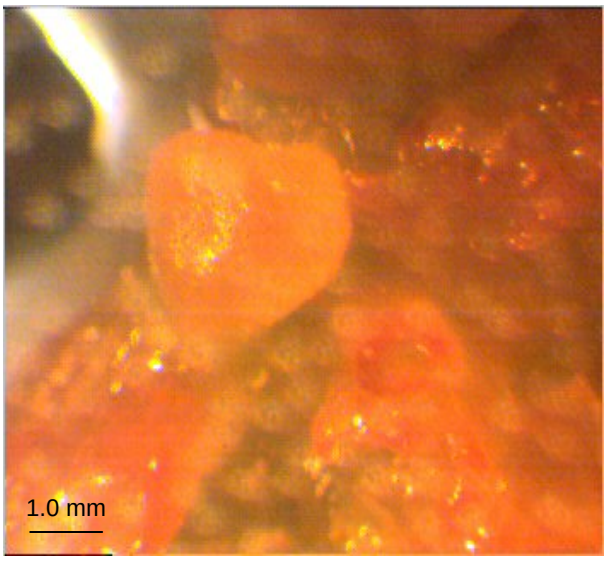


Plate 4.5: The heart-shaped stage of the somatic embryo derived from suspension cultures in the development media containing 1.0 mg/l BAP and 1.0 mg/l NAA after 8 weeks of culture.

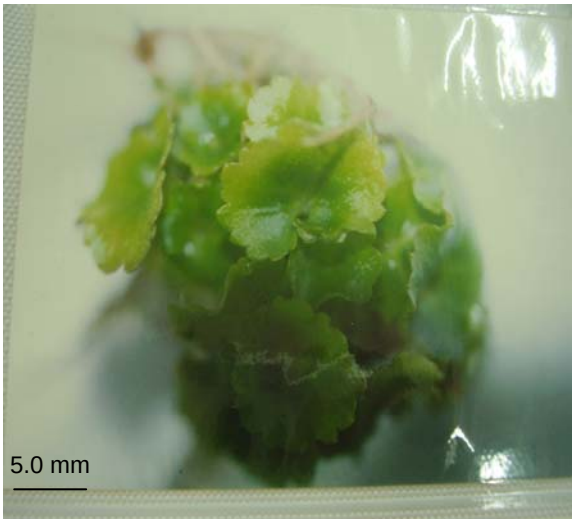


Plate 4.6: Multiple shoots formed from suspension cultures of *Begonia* x *hiemalis* Fotsch. placed on MS medium supplemented with 1.0 mg/l BAP, 1.0 mg/l NAA, 3.0% sucrose and 0.2% gelrite after 8 weeks of culture.

4.3.6 Scanning Electron Micrographs of Different Stages of Somatic Embryos

Using scanning electron microscope (SEM), different stages of somatic embryos were observed developing from a mass of globular structures on the surface of leaf section after 10 weeks of incubation. During the process of somatic embryogenesis, the different stages of somatic embryos developed starting from globular, heart-shaped, torpedo-shaped and lastly followed by cotyledonary-stage. The length of each embryo-stage range from 400-700 μm (refer to Plate 4.3-4.7). The emerging globular cells started to split and form a curve at the end of one side and developed into heart-shaped stage (Plate 4.4 and 4.5).

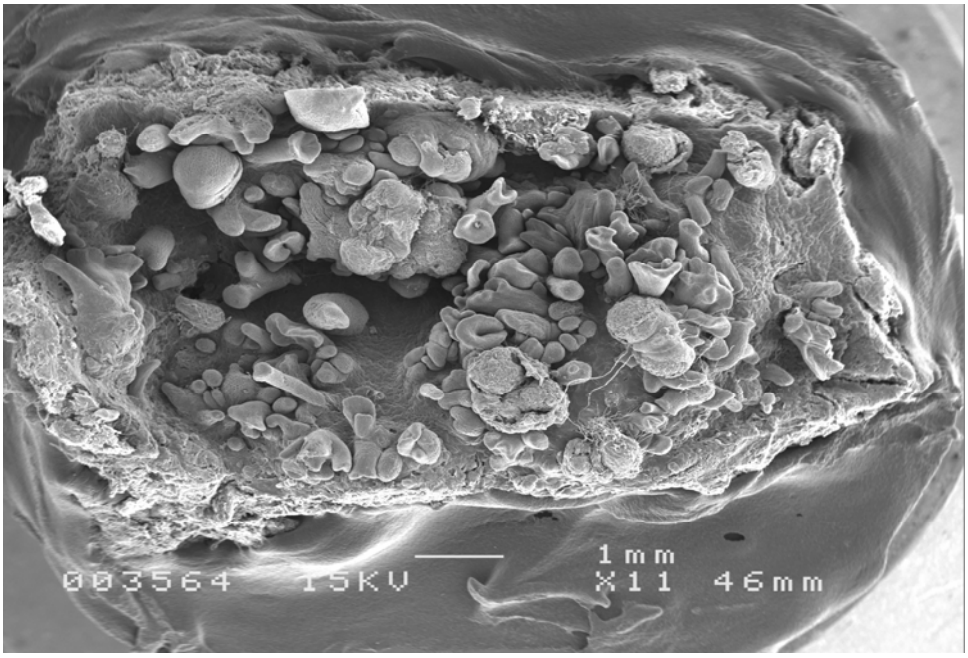


Plate 4.7: Scanning electron micrograph showing somatic embryogenesis in *Begonia* x *hiemalis* Fotsch. *in vitro*. Bar in each figure represents 1 mm.

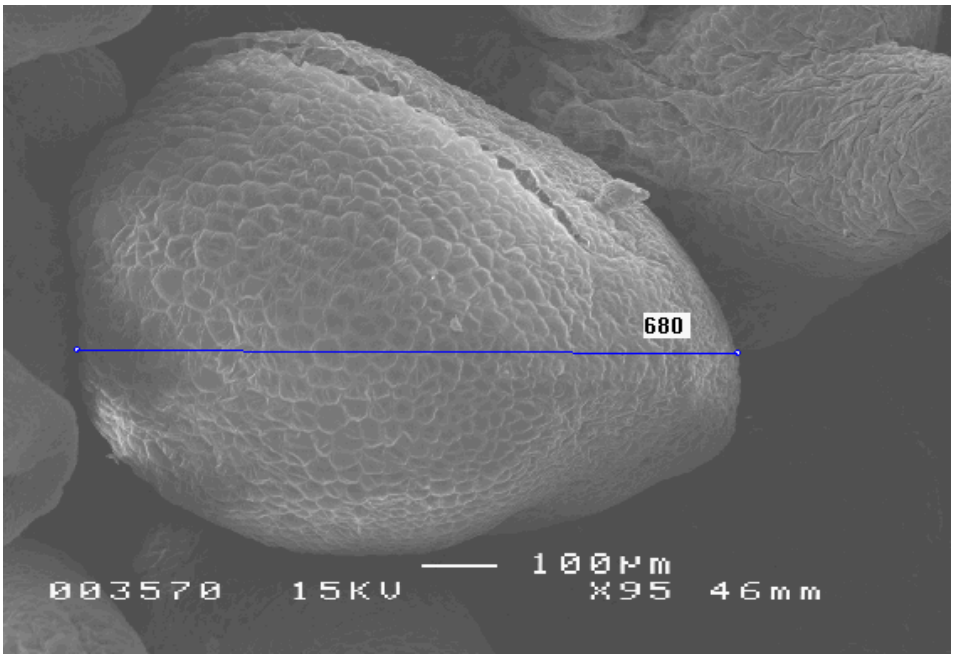


Plate 4.8: A globular stage of somatic embryo of *Begonia* x *hiemalis* Fotsch. Bar in each figure represents 100 µm.

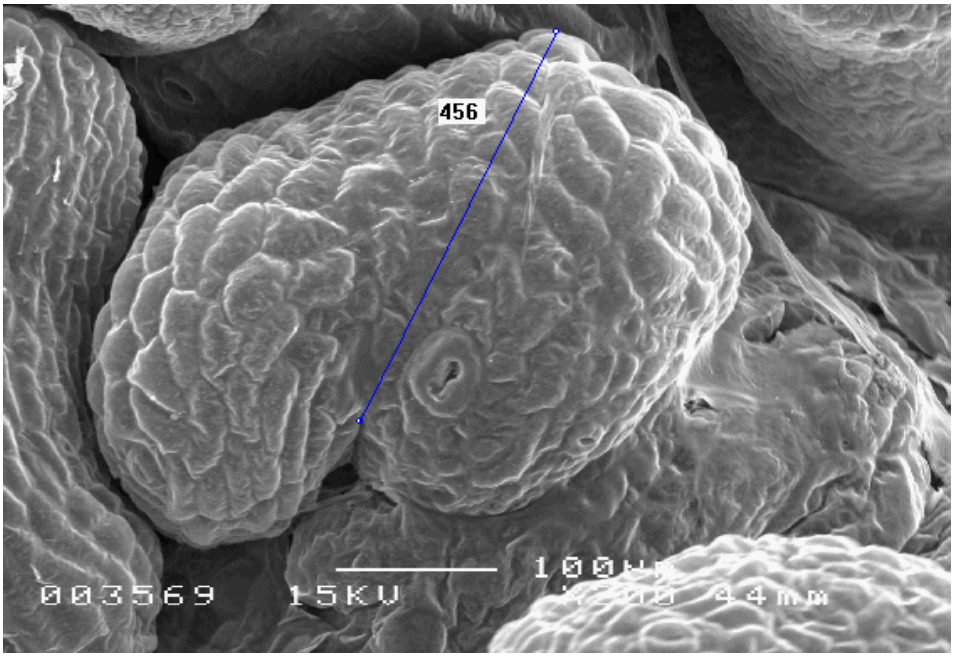


Plate 4.9: A heart-shaped stage of somatic embryo of *Begonia* x *hiemalis* Fotsch. Bar in each figure represents 100 µm.

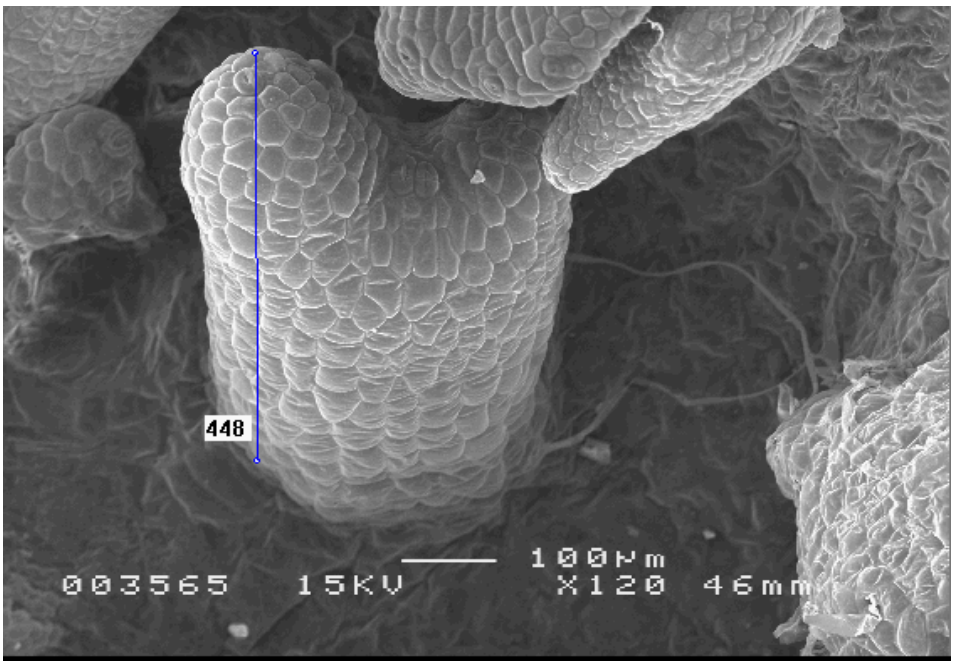


Plate 4.10: A torpedo-shape of somatic embryo of *Begonia* x *hiemalis* Fotsch. Bar in each figure represents 100 µm.

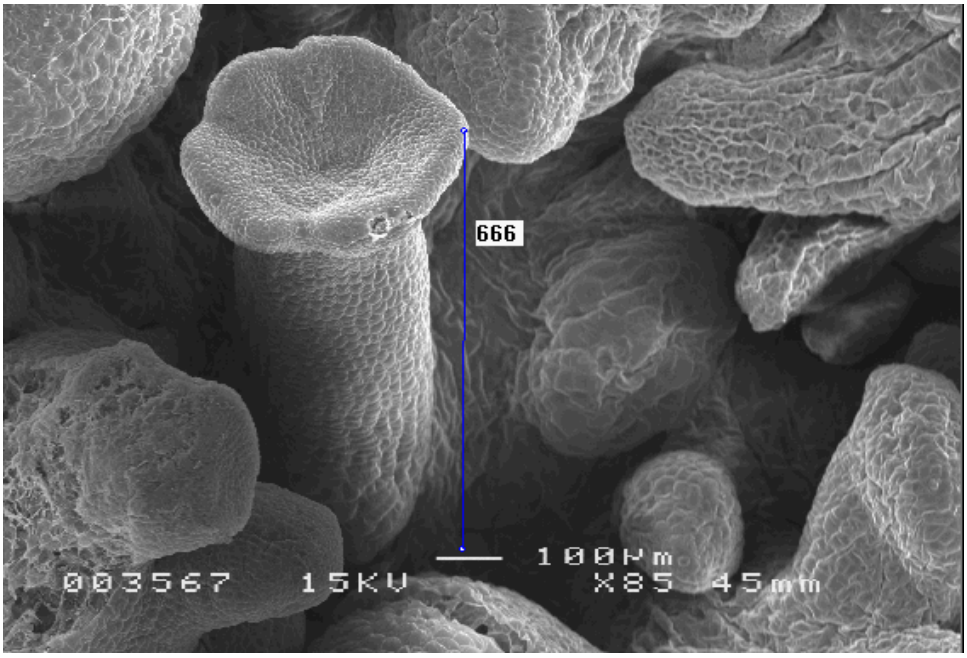


Plate 4.11: A cotyledonary stage of somatic embryo of *Begonia* x *hiemalis* Fotsch. Bar in each figure represents 100 µm.

4.4 SUMMARY

1.0 From the present investigation, the optimum medium that has been used for somatic embryo induction in Begonia was MS supplemented with 1.0 mg/l BAP, 0.1 mg/l 2,4-D and 500 mg/l casein hydrolysate.

2.0 Two to four-month-old plantlets were chosen as the explants for early cultures of somatic embryos due to their optimum response for regeneration.

3.0 The second to fifth position of leaf explants were identified as the most responsive explants.

4.0 MS supplemented with different concentrations of 2,4-D and TDZ applied singly could not result in direct somatic embryogenesis formation from leaf and petiole explants.

5.0 The plantlets after being treated with 1.0 mg/l TIBA gave high response in the induction and development of somatic embryogenesis as compared with the untreated plantlets. However, the growth rate of the plantlets that was treated with TIBA was less vigorous than the untreated plantlets.

6.0 Amongst all additives used for somatic embryogenesis induction in Begonia such as L-Proline, TIBA and sucrose, casein hydrolysate was identified as the best supplement for the process.

7.0 The 2 month-old plantlets that were grown on MS medium containing 1.0 mg/l TIBA produced abnormal growth as compared to those of the control medium. The anti-auxin property of TIBA disturbing the internal hormonal balance and the plantlets showed curved leaves and their roots revealed a negative geotropism and grew upward.

8.0 The upper surface of the explants that were incubated in the 24 darkness developed globular, heart-shaped, torpedo-shaped and cotyledonary-stage in somatic embryogenesis whereas under 16 hours light and 8 hours dark explants developed only globular, torpedo-shaped and cotyledonary-stage after 8 weeks of incubation.

CHAPTER 5

SYNTHETIC SEED PRODUCTION IN *Begonia* x *hiemalis* Fotsch.

5.1 EXPERIMENTAL AIMS

Synthetic seed technology is an alternative method to traditional micropropagation for production and delivery of cloned plantlets (Brishia *et al*., 2002). The actual form of synthetic seed (i.e., presence or absence of a synthetic seed coat, whether they are hydrated or dehydrated, quiescent or not, etc.) may vary depending on the specific crop application (Gray *et al*., 1995). Synthetic seeds are very useful for plants which do not produce viable true seeds. Research on synthetic seeds have been extended to numerous plants, including *Saintpaulia ionantha* Wendl. (Daud, 2006), *Syringa vulgaris* L.(Refouvelet *et. al*., 1998), *Solanum tuberosum* L. (Sarkar and Naik, 1998), *Asparagus cooperi* (Ghosh and Sen, 1994) strawberry and raspberry (Lisek and Orlikowska, 2004) and others.

Encapsulation of propagules (micro shoots, somatic embryos etc.) may provide physical protection. Ghosh and Sen (1994) also reported the advantages of synthetic seeds which include ease of handling and potential for long-term storage. Since medium or long term storage is one of the aims of synthetic seed production, their storage life is a critical parameter (Janeiro *et al*., 1997). Cold storage of encapsulated embryos is also important for post-storage survival and germination (Ipekci and Gozukirmizi, 2003).

To date, there has been no publication reporting the production of synthetic seeds from micro shoots and somatic embryos of *Begonia* x *hiemalis* Fotsch cv. *Schwabenland Red*. As techniques for somatic embryogenesis have improved, the possibility of encapsulation of somatic embryos for synthetic seed production was explored (Ipekci and Gozukirmizi, 2003). Synthetic seeds production of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red* was carried out using alginate-mediated, nutrient-encapsulated *in vitro* micro shoots and somatic embryos of this species.

The aim of this research was to develop an efficient procedure for the preparation of synthetic seeds from micro shoots and somatic embryos. The study also focused on the effect of different treatments of encapsulation matrix of micro shoots and somatic embryos. By using different concentrations of sodium alginate and different exposure to matrix solution, the optimum production of synthetic seeds could be obtained. In addition, the effect of storage period at 4^{o} C, germination potential and regeneration of the synthetic seeds *in vivo* and *in vitro* were also investigated.

5.2 MATERIALS AND METHODS

5.2.1 Explants for Propagule Preparation

The leaf explants were approximately cut into 5.0-10.0 mm segments and cultured onto MS medium supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA for shoot induction. The cultures were incubated in the culture room for 8 weeks after which the micro shoots were subcultured onto MS medium supplemented with 0.5 mg/l GA_3 for plantlet development. Prior to propagule preparation, the plant stocks were maintained in the culture room. *In vitro* plantlets were selected for propagule preparation rather than intact plants. This will help the mass propagation of sterile plantlets and prevent contaminations during the culture period.

Standard tissue culture methods were used in this study. The explants sources consisting of small pieces of leaves derived from *in vitro* plantlets were established on MS (Murashige and Skoog, 1962) medium supplemented with 3.0% (w/v) sucrose and 0.8% (w/v) technical agar fortified with 1.0 mg/l BAP and 1.0 mg/l NAA. The pH of the medium employed in the experiment was adjusted to 5.8 prior to sterilization process at 121 °C, 105 kPa for 20 minutes. Cultures were kept in the culture room at 25 ± 1 °C, under 16 hours light photoperiod of light intensity (1 000 Lux). Micro shoots (approximately 5.0 mm in length) were excised from cultures after 8 weeks in culture. The micro shoots were carefully isolated and were blotted dry on filter paper (Plate 6.1).

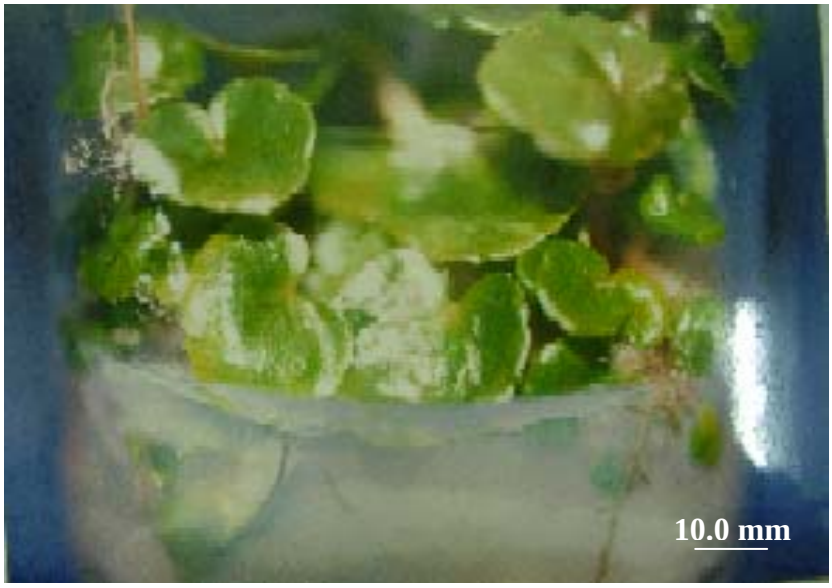


Plate 5.1: Axenic plants of *Begonia* x *hiemalis* Fotsch. after being cultured on MS supplemented with 0.5 mg/l GA_3 and 0.2% charcoal for 2 months were used for micro shoots induction.

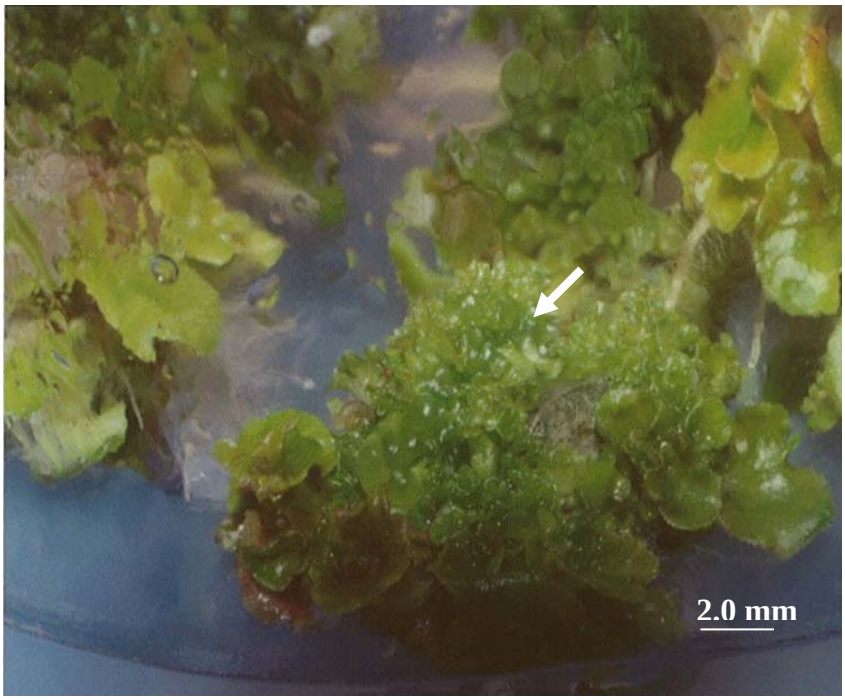


Plate 5.2: Micro shoots derived from leaf explants of *Begonia* x *hiemalis* Fotsch. after 8 weeks being cultured on MS supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA after 8 weeks of culture.

5.2.2 Preparation of Encapsulation Matrix

The encapsulation matrix was prepared by using MS medium free of calcium, supplemented with optimum regeneration hormones (1.0 mg/l BAP and 1.0 mg/l NAA), 3.0% (w/v) sucrose and 3.0% (w/v) sodium alginate. In most synthetic seeds studies, sodium alginate was used as a matrix for encapsulation process. Previous studies on synthetic seeds production also showed that the standard MS basal medium supplemented with sodium alginate have been widely used.

5.2.3 Formation of Synthetic Seeds

The ideal procedure for encapsulation of synthetic seeds were identified by studying the effect of various factors on synthetic seed formation which include: (1) concentration of sodium alginate, (2) concentration and duration of exposure to Calcium chloride ($CaCl_2.2H_2O$) solution, (3) presence or absence of hormone in the encapsulation solvent, (4) different types of sowing media and (5) storage period of the artificial seeds.

Micro shoots with distinct shoot and root meristems derived from organogenesis and somatic embryogenesis processes were selected to identify the ideal concentration of alginate for encapsulation. Micro shoots with maximum 3.0-5.0 mm length and mature stage embryos (cotyledonary-stage) of *Begonia* x *hiemalis* Fotsch. were taken from optimum regeneration media. Micro shoots were obtained from MS medium

supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA, whereas cotyledonary-stage embryos were developed from young leaf cultured on MS medium supplemented with 1.0 mg/l BAP, 0.1 mg/l 2,4-D and 500 mg/l casein hydrolysate. For the production of synthetic seeds in *Begonia* x *hiemalis* Fotsch., micro shoots or cotyledonary-stage of somatic embryos were mixed in the encapsulating matrix which consisted of 2.0-5.0% solutions of sodium alginate (Sigma), mixed with MS basal medium solution (pH 5.8) added with 3.0% (w/v) sucrose and 1.0 mg/l BAP in combination with 1.0 mg/l NAA.

Apart from that, the micro shoots were also encapsulated with sodium alginate solution devoid of MS basal and plant growth regulator. Subsequently, by using sterile micropipette, the micro shoots were drawn up with some encapsulation matrix and dropped into the matrix solution ($CaCl_2.2H_2O$ solution). Different concentrations of $CaCl_2.2H_2O$ solution (0.1- 1.0M) were also tested. The seeds were left to harden for a certain period (10-30 minutes) for complexation. Then, the seeds were washed in MS standard liquid medium to avoid from sticking together and retrieved using nylon mesh. The resulting capsules or beads consisted of one propagule per bead.

5.2.4 Germination Medium and Storage Period

The beads were germinated on various germination media and substrates, for germination evaluation. Germination media including MS basal media supplemented with 3.0% (w/v) sucrose, topsoil 1, vermiculite and also sphagnum peat. Topsoil 1 is the normal conventional soil containing a mix of 2 peat:1 soil:1 sand (by volume) fed with 10N-22.7P-

8.3K fertilizer (0.9 g/l). Sphagnum was bought from local plant nursery in Sungai Buloh, Selangor. Vermiculite is a natural mineral that is formed by hydration of certain basaltic minerals and was obtained from Sigma-Aldrich Company. Sphagnum and vermiculite were also fed with 10N-22.7P-8.3K fertilizer (0.9 g/l). All the germinating substrates were prepared in the sterile container and autoclaved prior to use.

The beads derived from micro shoots and somatic embryo explants were also cold-stored in the incubator at 4 °C from one to six months prior to germination process. The beads were blotted dry on autoclaved filter or tissue papers prior to culture and kept in the sterile petri dishes to avoid contaminations prior to storage process. All the samples derived from different explants consists of 30 beads were sown in MS basal medium and then incubated under the culture room conditions at 25 ± 1 °C, under 16 hours light photoperiod with light intensity of 2 000 Lux. The germination process of the synthetic seeds was recorded manually and germination rate were recorded after 6 weeks of sowing.

Non-encapsulated micro shoots and somatic embryos were used as controls. Control and encapsulated micro shoots or somatic embryos were placed in the sterile containers containing 50.0 ml of germination medium (MS basal medium). The cultures were incubated for 8 weeks in the culture room at 25 ± 1 °C, under 16 hours light photoperiod with light intensity of 2 000 Lux.

5.2.5 Statistical Analysis

All data and variables were statistically analyzed using SPSS statistical package version 11. Values are presented as mean ± SE. Mean percentage and Multiple Range Analysis were done on all data, using 95% LSD intervals method.

5.3 RESULTS

5.3.1 Formation of Synthetic Seeds

In this study, synthetic seeds were created from micro shoots and somatic embryos at cotyledonary phase. An assessment of various concentrations of sodium alginate (2.0-5.0%) and calcium chloride (0.25-1.25M) on bead formation is presented in Table 5.1. Micro shoots were successfully encapsulated in 3.0% (w/v) solution of sodium alginate and 1.0M of $CaCl_2.2H_2O$ that gave optimal beads with diameters ranging from 0.50-0.70 cm. The alginate solution prepared in MS salt solution together with 3.0% (w/v) sucrose was left to harden for another 30 minutes in 1.0M $CaCl_2.2H_2O$, which produced beads with firm coats, round beads and uniform size and in good shape for handling (Plate 5.3).

Lower concentrations of sodium alginate (1.0-2.0%) formed fragile beads with no definite shapes. At higher concentrations (more than 4.0%) the beads were isodiametric in shape but too hard. Observations were made after 30 minutes in $CaCl_2.2H_2O$ solution for hardening process. However, it was found that encapsulated micro shoots showed different degree of successes based on ideal beads produced. Further experiments were carried out in order to determine the optimal concentration of encapsulation matrix.

The results showed that the gel encapsulated the micro shoots was most effective when 3.0% (w/v) sodium alginate was used. Gel capsules formed with 2.0% (w/v) sodium alginate produced soft beads and difficult to handle, whereas, 5.0% (w/v) sodium alginate

Table 5.1: Effect of different concentrations of Sodium alginate and Calcium chloride on bead formation. Observations were taken after 30 min of complexation process in $CaCl_2.2H_2O$ solution.

$CaCl_2.2H_2O$	Sodium alginate concentration (%)				
(M)	**2.0**	**3.0**	**4.0**	**5.0**	**6.0**
0.25	+	+ + +	+ + +	+ + +	+ + +
0.50	+	+ + +	+ + +	+ + +	+ + +
0.75	+ +	+ + +	+ + +	+ + +	+ + + +
1.00	+ +	+ + + +	+ + + +	+ + + +	+ + + +
1.25	+ +	+ + + +	+ + + +	+ + + +	+ + + +

\+ Very fragile bead with no definite shape
+ + Fragile beads with no definite shape
+ + + Soft, solid and uniform shape
+ + + + Optimal, firm, uniform and round shape

Plate 5.3: Different structures of beads coated with different concentrations of sodium alginate (A) 2.0%, (B) 3.0%, (C) 4.0% and (D) 5.0%.

produced viscous and hard beads and prevented the emergence of shoots and roots during germination. In the present study, it was observed that MS medium containing 3.0% sodium alginate solution supplemented with 3.0% sucrose, 1.0 mg/l BAP in combination with 1.0 mg/l NAA maintained in 1.0 M $CaCl_2.2H_2O$ solution for encapsulation was the optimal concentration of complexation to produce artificial seeds from encapsulated micro shoots. The beads started to germinate after 7 days in culture with 90.48% germination rate and developed into plantlets 10 days later (Plate 5.4).

5.3.2 Germination of Synthetic Seeds *In Vitro*

The synthetic seeds could only germinate under aseptic conditions (Plate 5.4). The optimum germination rate of synthetic seeds from micro shoots under sterile conditions was 90.48% within one month of culture (Table 5.2), whereas, non-encapsulated micro shoots gave 80.00% germination on MS medium. For micro shoots encapsulated with sodium alginate matrix without sucrose, the percentage of germination was reduced to 80.95 % within 10 days of culture and the survival rate of the germinated seeds further reduced to 30.00% within one month incubation. Micro shoots, which were encapsulated with sodium alginate matrix with sucrose, gave 90.48% germination but the percentage was reduced to 64.00% after one month of culture. The overall percentage of germination of all types of encapsulated micro shoots was less compared to those encapsulated with sodium alginate matrix with sucrose, 1.0 mg/l BAP and 1.0 mg/l NAA (Table 5.2).

Table 5.2: Growth response of micro shoots and somatic embryos of *Begonia* x *hiemalis* Fotsch. encapsulated in different capsule matrix after being transplanted onto MS media for 10 and 30 days. Non-encapsulated micro shoots and somatic embryos were used as controls.

Capsule matrix	Germination rate (after 10 days) (% ± SE)	Survival rate (after 30 days) (% ± SE)
Micro shoots		
Control	80.00 ± 0.08 $_{b}$	80.00 ± 0.08 $_{a}$
Ca-free MS + distilled water	80.95 ± 0.06 $_{b}$	30.00 ± 1.58 $_{c}$
Ca-free MS + 3% sucrose	90.48 ± 1.08$_{a}$	64.00 ± 1.20 $_{b}$
Ca-free MS + 3% sucrose + 1.0mg/l BAP + 1.0mg/l NAA	90.48 ± 1.08 $_{a}$	83.33 ± 0.06 $_{a}$
Somatic embryos		
Control	80.00 ± 0.08 $_{a}$	80.00 ± 0.08 $_{a}$
Ca-free MS + distilled water	80.95 ± 0.06 $_{a}$	30.00 ± 1.58 $_{d}$
Ca-free MS + 3% sucrose	80.95 ± 1.08 $_{a}$	50.00 ± 1.20 $_{c}$
Ca-free MS + 3% sucrose + 1.0mg/l BAP + 1.0mg/l NAA	85.00 ± 1.08 $_{a}$	76.67 ± 0.06 $_{b}$

* Figure followed by the same letter in the columns are not significantly different at P<0.05.

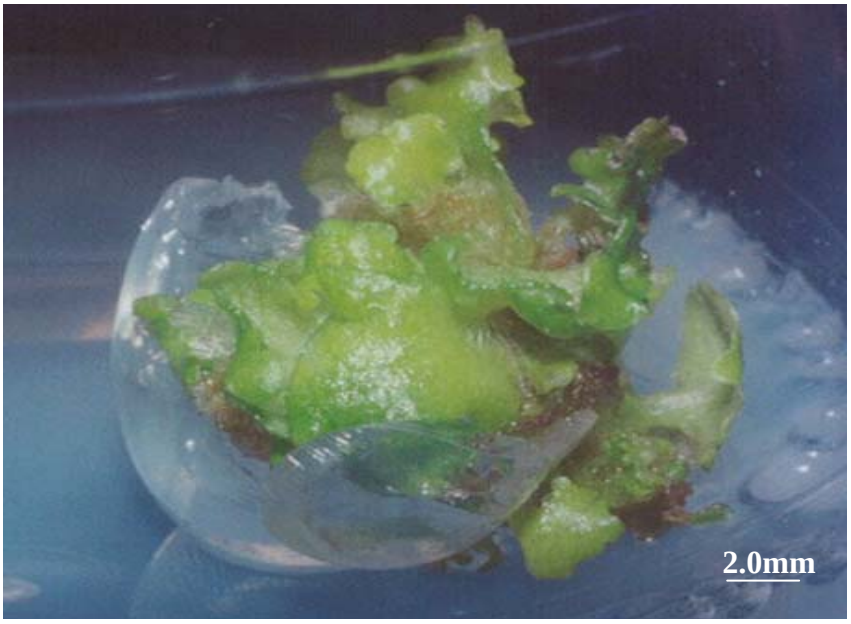


Plate 5.4: The emergence of shoot from synthetic seed after being germinated on MS basal media for 2 weeks.

Plate 5.5: The synthetic seed of *Begonia* x *hiemalis* Fotsch. germinated on vermiculite after 12 weeks.

The percentage of germination on MS medium for somatic embryos encapsulated with alginate matrix prepared with sucrose, 1.0 mg/l BAP and 1.0 mg/l NAA was 80.00% (Table 5.2), whereas, somatic embryos, which were encapsulated with sodium alginate matrix with sucrose, gave 80.95% germination but the percentage was reduced to 50.00% after one month of culture. For somatic embryo encapsulated with sodium alginate matrix without sucrose, the percentage of germination was 80.95 % within 10 days of culture and the survival rate of the germinated seeds reduced to 30.00% within one month incubation.

Synthetic seeds with no sucrose in the capsule had significantly lower germination rates than encapsulated micro shoots or somatic embryos as compared with synthetic seeds with sucrose. Overall the germination of different types of encapsulated matrix for somatic embryos was lower than micro shoots on MS medium. However, the optimum germination of the seeds was obtained in sodium alginate matrix with sucrose, 1.0 mg/l BAP and 1.0 mg/l NAA. Thus, in all subsequent experiments, alginate matrix prepared with sucrose, 1.0 mg/l BAP and 1.0 mg/l NAA was used as the complexation matrix for the beads production as it was proved to be the most suitable complexation medium.

5.3.3 Germination of Synthetic Seeds *In Vivo*

The encapsulated explants were also germinated under *in vivo* conditions on topsoil 1, vermiculite and sphagnum (Table 5.3). The evaluation of bead germination includes observation in the increase of size of explants, ability to break the capsule and extrusion of

Table 5.3: Effect of different sowing media on germination rate of synthetic seeds of *Begonia* x *hiemalis* Fotsch.. Non-encapsulated micro shoots and somatic embryos were used as controls.

Sowing medium	No. of synthetic seeds	No. germinated	Germination rate (% ± SE)
Micro shoots			
Control	30	15	$50.00 \pm 0.08_{c}$
Topsoil 1	30	25	$83.33 \pm 0.06_{a}$
Vermiculite	30	17	$56.67 \pm 1.20_{b}$
Sphagnum	30	11	$36.67 \pm 1.18_{d}$
Somatic embryos			
Control	30	10	$33.33 \pm 0.10_{c}$
Topsoil 1	30	20	$66.67 \pm 0.06_{a}$
Vermiculite	30	15	$50.00 \pm 1.50_{b}$
Sphagnum	30	10	$33.33 \pm 1.1_{c}$

* Figure followed by the same letter in the columns are not significantly different at $P<0.05$.

Plate 5.6: Plantlet regenerated from synthetic seed after 2 months (A) and produced flower after 6 months transferred to soil (B).

the shoot or of a leaf bud. The explants were considered alive if they remained green, with no necrosis or yellowing and continued to enlarge after encapsulation.

Most of the micro shoot beads managed to germinate on MS basal medium (50.00%) and sterile topsoil 1 (83.33%). The percentage of germination for beads on vermiculite was 56.67% and encapsulated micro shoots showed 36.67% when placed on sphagnum. The survival rate of seedlings reached 80.00% after they were transferred to pots (Plate 5.6). However, the germination percentage was lower when encapsulated somatic embryos were sown on topsoil 1, vermiculite and sphagnum. The results showed that 66.67% germination rate could be obtained from topsoil 1 and 50.00% germination obtained from vermiculite, whereas only 33.33% of encapsulated somatic embryos managed to germinate on sphagnum.

5.3.4 The Effect of Storage Period on Germination Capability of The Seeds

Plantlet regeneration from cold-stored (4 °C) synthetic seeds of micro shoots and somatic embryos was similar compared to regeneration from normal cultures (25 °C). The results showed that 100.00% germination rate of encapsulated micro shoots could be obtained from one to two months storage, whereas three months storage also showed precocious germination and the seeds germinated at 83.33%. The four and six months storage seeds only showed 53.33% and 6.67% germination respectively. After one month's storage at low temperature (4 °C), the viability of seeds had fallen from 100.00% to 96.67 % after being cultured onto germination medium. Encapsulated micro shoots retained its ability to

germinate (83.33%) and developed shoots even when stored up to 90 days. After 120 days of storage, the germination percentage of encapsulated micro shoots was 50.00% but the seeds have lost the viability to generate into seedlings.

Somatic embryos encapsulated with 3.0% sodium alginate also gave 100.00% germination without storage (Table 5.4). After one month's storage at low temperature (4 °C), the viability of seeds had fallen from 100.00% to 90.48 % after being cultured onto germination medium. Encapsulated somatic embryos retained their ability to germinate and develop shoots even when stored up to 90 days. After 120 days of storage, the germination percentage of encapsulated somatic embryos was 6.67% and failed to show any potential of germination.

Most of the synthetic seeds derived from somatic embryos developed into normal plants and exhibited similar traits as the encapsulated somatic embryos. No phenotypic changes were observed in the plantlets and over 90.00% of the plantlets developed into healthy, fully-grown plants with about 5.0 cm height after 4 months cultured onto MS basal medium.

Table 5.4: Effect of storage time (days) on germination of synthetic seeds on MS basal medium at 4°C.

Storage period (days)	No of synthetic seeds	No. germinated	Germination rate (% ± SE)
Micro shoots			
0	30	30	$100.00 \pm 0.00_{a}$
30	30	29	$96.67 \pm 0.0_{b}$
60	30	30	$100.00 \pm 0.00_{a}$
90	30	25	$83.33 \pm 0.06_{c}$
120	30	16	$53.33 \pm 1.00_{c}$
150	30	2	$6.67 \pm 0.00_{d}$
180	30	0	0_{e}
Somatic embryos			
0	30	30	$100.00 \pm 0.00_{a}$
30	30	29	$90.48 \pm 1.08_{b}$
60	30	25	$83.33 \pm 0.06_{c}$
90	30	15	$50.00 \pm 1.00_{d}$
120	30	2	$6.67 \pm 0.00_{e}$
150	30	0	0_{e}
180	30	0	0_{e}

* Figure followed by the same letter in the columns are not significantly different at $P<0.05$.

5.3 SUMMARY

1. Synthetic or artificial seeds were successfully produced in the present work.

2. Micro shoots and somatic embryos were successfully encapsulated in 3.0% (w/v) sodium alginate. Lower concentrations of sodium alginate (1.0-2.0%) formed soft, fragile beads, whereas higher concentrations (more then 4.0%) produced solid and hard beads which made germination difficult i.e. difficult for the emergence of the propagules.

3. The 3.0% (w/v) alginate solution containing MS salt together with 3.0% (w/v) sucrose which was left to harden for another 30 min. in 1.0M $CaCl_2.2H_2O$ produced ideal beads formation which are isodiametric in shape, firm and clear with diameters ranging from 0.50-0.70 cm.

4. The beads were successfully germinated on MS basal medium devoid of plant growth regulators, topsoil 1 and vermiculite, whereas low germination rate was obtained using sphagnum as a sowing medium.

5. The encapsulated micro shoots and somatic embryos germinated on MS basal medium, topsoil 1, vermiculite and sphagnum within 7-10 days.

6. The growth rate of the synthetic seeds of micro shoots on MS basal medium was 100.00%, 83.33% in sterile topsoil 1, 56.67% in sterile vermiculite and 36.67% in sterile sphagnum.

7. The growth rate of the synthetic seeds of somatic embryos on MS basal medium was 100.00%, 66.67% in sterile topsoil 1, 50.00% in sterile vermiculite and 33.33% in sterile sphagnum.

8. The synthetic seeds of micro shoots stored 30-90 days at 4 °C were successfully germinated on MS basal medium devoid of plant growth regulators. The germination rate was 96.67-83.33% respectively. Germination occurred within 10 days.

9. The synthetic seeds of somatic embryos stored 30-90 days at 4 °C were successfully germinated on MS basal medium devoid of plant growth regulators. The germination rate was 90.48-50.00%. Germination occurred within 10 days.

10. The germination rate of the encapsulated micro shoots after being stored at 120 days was 53.00% and 6.67% for encapsulated somatic embryos but they failed to show any potential to generate.

CHAPTER 6

IN VITRO FLOWERING OF *Begonia* x *hiemalis* Fotsch.

6.1 EXPERIMENTAL AIMS

Begonia x *hiemalis* Fotsch. cv. *Schwabenland Red*, known as Begonia Rose is primarily grown as ornamental plant throughout the temperate regions of the world and being produced in the developed countries as a flowering potted plants. However, under the greenhouse conditions, the conventional methods of propagation are problematic due to rapid occurrence of diseases. The production of large numbers of genetically homogenous plant is also very difficult (Rout *et al*., 2006). Begonia is a short day plant and the time of bud appearance or flowering induction was strongly affected by day length as well as by night temperature (Heide, 1962). Flowering in Begonia may also be affected due to these factors and to keep in pace with the increasing demands of Begonia as a flower potting plants, methods for rapid *in vitro* flowering have been developed in this study.

The timing of the transition from vegetative growth to flowering is of paramount importance in agriculture, horticulture and plant breeding because flowering is the first step of sexual reproduction (Bernier *et al.*, 1993). Under natural growth, flower formation usually commences when a plant attains maturity (Virupakshi *et al*., 2002). *In vitro* flowering serves as an important tool to study flower induction and initiation, and floral development (Ziv and Naor, 2006). *In vitro* flowering induction involved various factors

that can be classified as internal and external factors such as media components, level of plant growth regulators and culture conditions. Mostly commonly used plant growth regulators for flowering *in vitro* are cytokinins, gibberellins and auxins (Ziv and Naor, 2006). Previous investigations also found that exogenous cytokinins significantly influences *in vitro* flowering in many plant species (Chang and Chang, 2003; Lin *et al*., 2003; Kostenyuk *et al.*, 1999). In addition to auxins and cytokinins, adenine is very important for the formation of flower buds on floral stalk explants (Ringe and Nitsch, 1968).

Consequently, there are a number of reports concerning plant regeneration from inflorescence in Begonia. However, there is no report concerning *in vitro* flowering from immature inflorescence of Begonia. Thus, *in vitro* flowering induction of Begonia was carried out in this study to obtain the best protocol for the production of flowers *in vitro*.

This study focused on the effect of different types of explants, growth regulators combinations, different concentrations sucrose, additive such as adenine and different dark incubation periods for optimizing the induction of *in vitro* flowering. Three different explants derived from reproductive organs of intact flowering plants were selected in this study i.e. young inflorescences, peduncles and petals. Ringe and Nitsch (1968) codded from previous research, reported that cuttings taken from flowering Begonias regenerated plants which flowered much earlier than plants regenerated from Begonias which were still vegetative.

Takimoto (1960) found that all the plants producing little chlorophyll initiated flower buds independently of the light conditions and the plants cultured on the sucrose-medium did not require high-intensity of light for flower initiation. Takimoto (1960) also suggested that the plants receiving sugar might require only long dark periods for flower initiation, the applied sugar replacing the high-intensity light process.

The present experiment was carried out in order to investigate whether it is possible to induce *in vitro* flowering during tissue culture and also during acclimatization period. The response to dark treatments, different sucrose concentrations and the application of additive such as adenine were compared in three different responsive explants i.e. flower peduncles, petals and young inflorescences. Thus, the applications of these explants for *in vitro* flowering purposes will benefit in producing early flowering without undergoing growth cycle of the selected plants.

6.2 MATERIALS AND METHODS

6.2.1 Plant Materials and Culture Conditions

Three-month-old of stock plants of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red* were obtained from local nursery in Sungai Buloh, Selangor and were maintained in controlled environment at 25 ± 1 ºC with a 16 hours photoperiod. For the purpose of *in vitro* flower induction, three different intact explants such as inflorescences, flower peduncles and flower petals from intact plants were utilized. In all cases, the stock plants were during flowering stages when the explants were taken from them. The inflorescences were harvested 4-5 days prior to the opening of the first flower.

The intact explants were washed with tap water for ½ to 1 hour. Under aseptic conditions, the explants were surface sterilized using 50.0% (v/v) sodium hypochlorite solution for 1 min. The explants were further washed three times with sterile distilled water and followed by 70.0% (v/v) of alcohol for 1 min. Finally, they were washed three times with sterile distilled water to remove the remaining alcohol.

The segments of flower peduncles, petals and inflorescences were then cut (5.0-10.0 mm in length) and grown onto solid medium which consisted of MS supplemented with selected growth regulators, chemicals such as adenine, 3.0% (w/v) sucrose and 0.8% (w/v) Agar Technical. The pH of the medium was adjusted to 5.8 prior to sterilization in the autoclave at 121 ºC and 103 kPa for 20 minutes. All the cultures were maintained under controlled

environments at 25 ± 1 ºC with a 16-hour photoperiod and irradiance of 1 000 Lux, supplied by Cool White fluorescent lamps. The *in vitro*-derived flowers were observed until 8 weeks of incubation and *in vitro*-derived plants were observed through at least one full flowering cycle to identify true-to type plants.

6.2.2 Screening for Suitable Explants for *In Vitro* Flowering Induction

Three different explants including inflorescences, flower peduncles and petals were excised from actively growing plants. The flower peduncles were approximately cut into 5.0-10.0 mm and petals were cut into 5.0 x 5.0 mm^2, whereas the basal part of the inflorescences were cut and the explants were cultured onto MS supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA, 3.0% (w/v) sucrose and 40 mg/l adenine for flower induction. The cultures were incubated in the culture room for 8 weeks.

6.2.3 Screening for Suitable Growth Regulators and Additive for *In Vitro* Flowering Induction

Prior to *in vitro* flowering induction, preliminary studies were done to obtain a good combination range between NAA and BAP for inducing the optimum response in three different explants. By using two combinations of BAP and NAA, the explants from intact plants were cultured onto the selected media. All media contained 3.0% (w/v) sucrose and 0.8% (w/v) Agar Technical. The pH was adjusted to 5.8 before autoclaving.

All the cultures were subjected to controlled environments at 25 ± 1 ºC with a 16-hour photoperiod and irradiance of 1000 Lux, supplied by Cool White fluorescent lamps. The macro-morphogenetic responses based on formation of vegetative and reproductive organs which were observed 8 weeks after the initiation of the cultures were identified and the numbers of regenerated shoots and flowers were scored from 8-12 weeks. The following are the list of MS media with different combinations of plant growth regulators, sucrose and adenine that were implemented in this study.

1. MS + 0.1 BAP + 0.1 NAA + 2.0% sucrose + 40 mg/l adenine
2. MS + 0.1 BAP + 0.1 NAA + 4.0% sucrose + 40 mg/l adenine
3. MS + 0.1 BAP + 1.0 NAA + 2.0% sucrose + 40 mg/l adenine
4. MS + 0.1 BAP + 1.0 NAA + 4.0% sucrose + 40 mg/l adenine
5. MS + 1.0 BAP + 1.0 NAA + 3.0% sucrose (control)
6. MS + 1.0 BAP + 0.1 NAA + 4.0% sucrose + 40 mg/l adenine
7. MS + 1.0 BAP + 1.0 NAA + 4.0% sucrose + 40 mg/l adenine

6.2.4 Screening for Suitable Sucrose Concentrations for *In Vitro* Flowering Induction

Different concentrations of sucrose for *in vitro* flowering induction were used on two different explants i.e. peduncle and inflorescence explants. Peduncle and inflorescence segments were cultured on the *in vitro* flowering media containing 40 mg/l of adenine and supplemented with different concentrations of sucrose. Five different concentrations of

sucrose were used in this experiment, including 1.0%, 2.0%, 3.0%, 4.0% and 5.0% (w/v). The pH of the media was adjusted to pH 5.8 prior to autoclaving. Each treatment was replicated twice. All cultures were maintained under controlled environments at 25 ± 1 ºC with a 16-hour photoperiod and irradiance of 1000 Lux, supplied by Cool White fluorescent lamps.

6.2.5 Screening for Suitable Adenine Concentrations for *In Vitro* Flowering Induction

In order to study the effect of different concentrations of adenine on *in vitro* flowering induction, 5 different concentrations of adenine were selected in this study. Different concentrations of adenine (20 mg/l, 40 mg/l, 60 mg/l, 80 mg/l and 100 mg/l) were supplemented together with the same concentration of sucrose (3.0%) and hormones i.e. 1.0 mg/l BAP and 1.0 mg/l NAA. Two different explants were selected i.e. peduncle and inflorescence explants. All the cultures were subjected to controlled environments at 25 ± 1 ºC with a 16-hour photoperiod and irradiance of 1000 Lux, supplied by Cool White fluorescent lamps.

6.2.6 Screening for Dark Incubation Period for *In Vitro* Flowering Induction

Different dark incubation periods were selected to study the effect of dark treatment on *in vitro* flowering induction. The peduncle and inflorescence explants were cultured on MS media supplemented with 1.0 mg/l BAP, 1.0 mg/l NAA, 3.0% (w/v) sucrose, 40 mg/l adenine and incubated for 2, 4, 6, 8, 10, 12, 14 days, 3 weeks and 4 weeks in the dark. The explants that were maintained in the dark were incubated in dark box in the growth chamber and the temperature was set to 25 ± 1ºC. The explants were incubated for 8 weeks and the observation was made at every one week interval.

6.2.7 *Ex Vitro* Flowering of The Plantlets

In vitro flowering induction from four different types of experiments (*in vitro* regeneration, suspension cultures, synthetic seeds production and somatic embryogenesis) were acclimatized in the green house. The plantlets were taken out from the containers and transferred into black polyethylene bags or plastic pots, measuring 20 x 25 cm^2, containing topsoil substrates after 4 months of incubation in the culture room. The acclimatized plantlets were transferred to the greenhouse and the fertilizer was applied once a week by using commercial fertilizer for further plant and floral growth and development.

6.2.8 Statistical Analysis

All data and variables were statistically analyzed using SPSS statistical package version 11. Values are presented as mean ± SE. One-way ANOVA and Multiple Range Analysis were done on all data, using 95% LSD intervals method.

6.3 RESULTS

Generally, *in vitro* flowering could be obtained when the inflorescence explants were cultured on MS supplemented with 1.0 mg/l BAP, 1.0 mg/l NAA, 3.0% (w/v) sucrose and 40 mg/l adenine after 8 weeks of culture. The results obtained from this chapter can be divided into two different categories i.e.(1) the induction of direct vegetative and reproductive shoot or floral bud from selected explants and, (2) the formation of floral bud from different types of plantlets. Apart from that, the effects of three different factors were also examined in promoting direct *in vitro* flowering induction including the effect of different concentrations of sucrose content, different concentrations of adenine and also different photoperiod. However, *ex vitro* flowering was obtained from different types of plantlets derived from *in vitro* regeneration, suspension cultures, synthetic seeds production and somatic embryogenesis experiments of previous studies. The results were elaborated in Part 6.3.6.

6.3.1 The *In Vitro* Flowering Induction from Different Explants

Three different types of explants were tested in this study, including flower inflorescences, peduncles and petals. Vegetative buds or micro shoots was observed, in both, the inflorescence and peduncle explants within 4 weeks of culture at all the growth regulators combinations (Table 6.1). Whereas, vegetative and reproductive buds were not obtained from petals cultured on MS medium supplemented with different concentrations of BAP (0.1 and 1.0 mg/l) and NAA (0.1 and 1.0 mg/l) (Table 6.1). The results also revealed that the petal explants could not produce direct *in vitro* flowering in Begonia. Macroscopic

observations showed that the explants become necrotic after 1 week of culture. The explants were then covered with proliferating callus. After 4 weeks, the petal explants were necrotic and did not develop into shoot even though they were subcultured onto regeneration medium. However, the young inflorescence and peduncle explants managed to give encouraging response such as formation of green and red micro shoots after 4 weeks of culture.

6.3.2 The Effect of Different Combinations of Growth Regulators on *In Vitro* Flowering

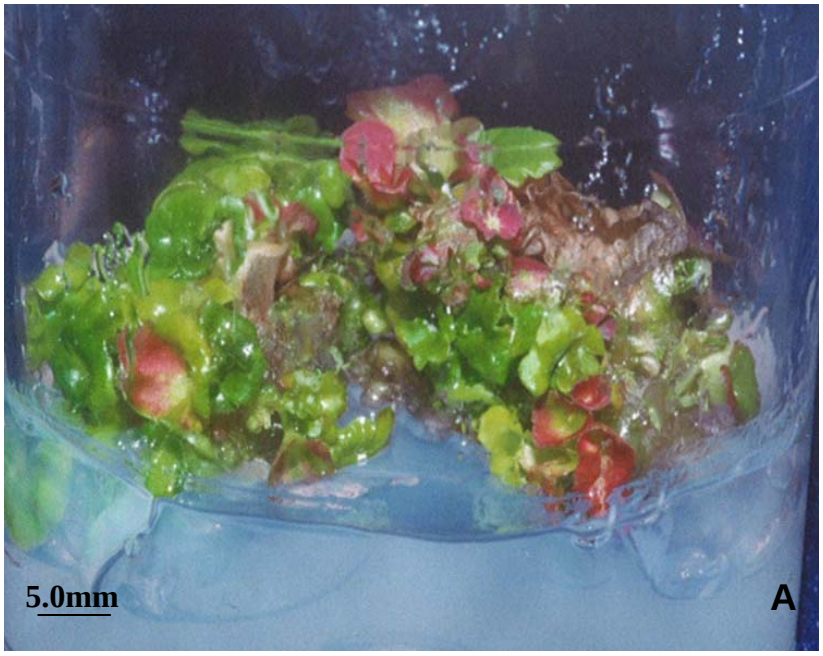
Although reproductive organs such as inflorescences, peduncles and petals were used as explants for *in vitro* flowering induction, most of the explants gave positive effect on vegetative shoot buds initiation. From observations it was found that vegetative shoot buds mostly initiated from two different types of explants i.e. flower peduncles and inflorescences segments of intact explants after 2 weeks of culture. The percentage of vegetative shoot buds ranged from 24.00% to 94.50% in inflorescence explants and from 5.00% to 40.00% in peduncles explants. A high frequency of vegetative shoot formation (94.50%) was observed on MS with 0.1 mg/l BAP in combination with 0.1mg/l NAA and supplemented with 4.0% (w/v) sucrose and 40 mg/l adenine (Table 6.1, Plate 6.1).

Table 6.1: Vegetative and reproductive shoot formation of *Begonia* x *hiemalis* Fotsch. on MS media supplemented with adenine, sucrose, combinations of BAP and NAA and incubated for 8 weeks at 25 ± 1 ºC with 16 hours light and 8 hours dark.

MS media				Observations	Vegetative shoots/explant (% ± SE)	Reproductive shoot formation (%± SE)
Growth regulators		Sucrose	Adenine			
1. Inflorescence						
0.1 BAP	0.1 NAA	2	40	Green normal shoots	66.00 ± 8.83 $_c$	**n.r.
0.1 BAP	0.1 NAA	4	40	Greenish normal shoots	94.50 ± 7.59$_a$	**n.r.
0.1 BAP	1.0 NAA	2	40	Red small shoots	79.00 ± 7.18 $_b$	**n.r.
0.1 BAP	1.0 NAA	4	40	Red small shoots	68.00 ± 1.52 $_c$	**n.r.
1.0 BAP	1.0 NAA	3	-	Green small shoots	24.00 ± 9.40 $_f$	**n.r.
1.0 BAP	1.0 NAA	3	40	Red small shoots	56.00 ± 9.95 $_d$	**n.r.
1.0 BAP	1.0 NAA	4	40	Green small shoots	50.00 ± 7.95 $_d$	**n.r.
2. Peduncle						
0.1 BAP	0.1 NAA	2	40	Greenish small shoots	5.00 ± 0.00$_c$	**n.r.
0.1 BAP	0.1 NAA	4	40	Greenish normal shoots	40.00 ± 5.62$_a$	**n.r.
0.1 BAP	1.0 NAA	2	40	Red small shoots	38.00 ± 7.68$_a$	**n.r.
0.1 BAP	1.0 NAA	4	40	Red small shoots	34.00 ± 1.95$_a$	**n.r.
1.0 BAP	1.0 NAA	3	-	Green small shoots	23.50 ± 8.75$_b$	**n.r.
1.0 BAP	1.0 NAA	3	40	Red small shoots	40.50 ± 9.99$_a$	**n.r.
1.0 BAP	1.0 NAA	4	40	Green small shoots	8.50 ± 2.35$_c$	**n.r.
3. Petal						
0.1 BAP	0.1 NAA	2	40	Red small shoots	**n.r.	**n.r.
0.1 BAP	0.1 NAA	4	40	Green small shoots	**n.r.	**n.r.
0.1 BAP	1.0 NAA	2	40	Red small shoots	**n.r.	**n.r.
0.1 BAP	1.0 NAA	4	40	Red small shoots	**n.r.	**n.r.
1.0 BAP	1.0 NAA	3	-	Green small shoots	**n.r.	**n.r.
1.0 BAP	1.0 NAA	3	40	Red small shoots	**n.r.	**n.r.
1.0 BAP	1.0 NAA	4	40	Greenish small shoots	**n.r.	**n.r.

* Mean values followed by the same letters within a column are not significantly different at the 0.05 level according to LSD test.

**n.r. = No response



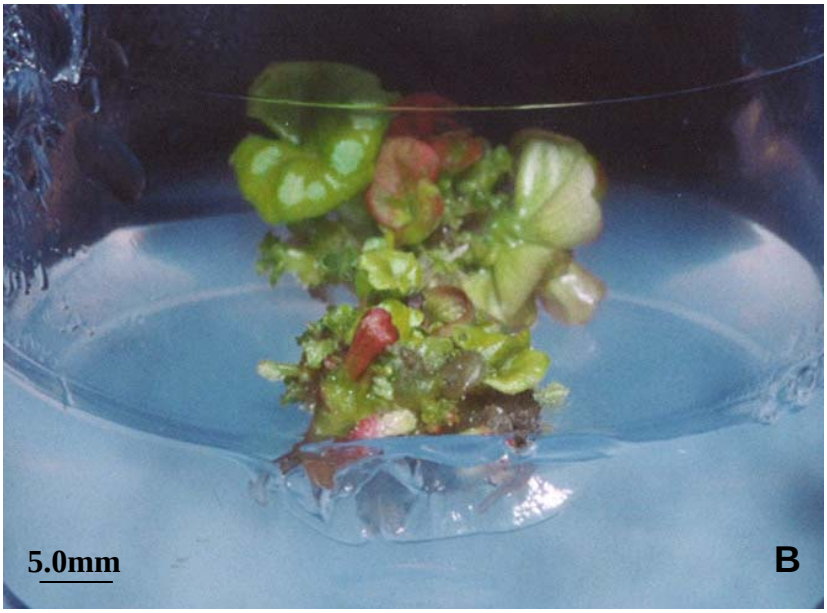
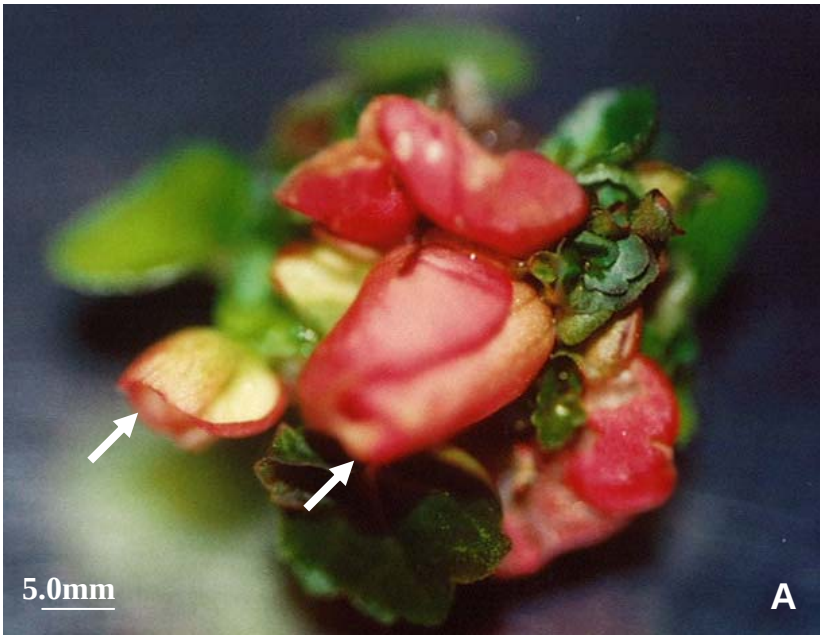


Plate 6.1: The formation of red coloured (A), abnormal vegetative shoots (B) from inflorescence explants, cultured on MS medium supplemented with adenine, combinations of BAP and NAA and incubated for 8 weeks at 25 ± 1 ºC with 16 hours light and 8 hours dark.

Even though petal explants managed to form vegetative shoot buds however, the chances was too low. When the whole inflorescences were cultured *in vitro*, plantlets grew from the axils of the bracts after 3-4 weeks of incubation. Subculturing with 2 weeks intervals of vegetative shoots onto MS supplemented with 1.0 mg/l BAP and 0.1 mg/l NAA combinations induced the plantlets production, reproductive bud and rooting from inflorescence explants.

The results showed that direct *in vitro* flowering or reproductive shoots were also obtained only from inflorescence explants cultured on MS medium supplemented with 1.0 mg/l BAP, 1.0 mg/l NAA, 4.0% (w/v) sucrose and 40 mg/l adenine. The floral buds bloomed after 6-8 weeks of incubation period. However, most macro-morphological structures of the flowers were abnormal whereby no reproductive parts such as stamen and carpel were developed in each floral organ except for the collectively sepals known as the calyx (Plate 6.2).

Developing inflorescences from young inflorescence explants were embedded within shoots. Even when explants were subcultured onto fresh media, containing MS basal media for 8 weeks, the flowers did not mature. Typically only one or two inflorescences were produced per flowering explant. At the end of 5 weeks incubation period, the plantlets were subcultured onto rooting media.



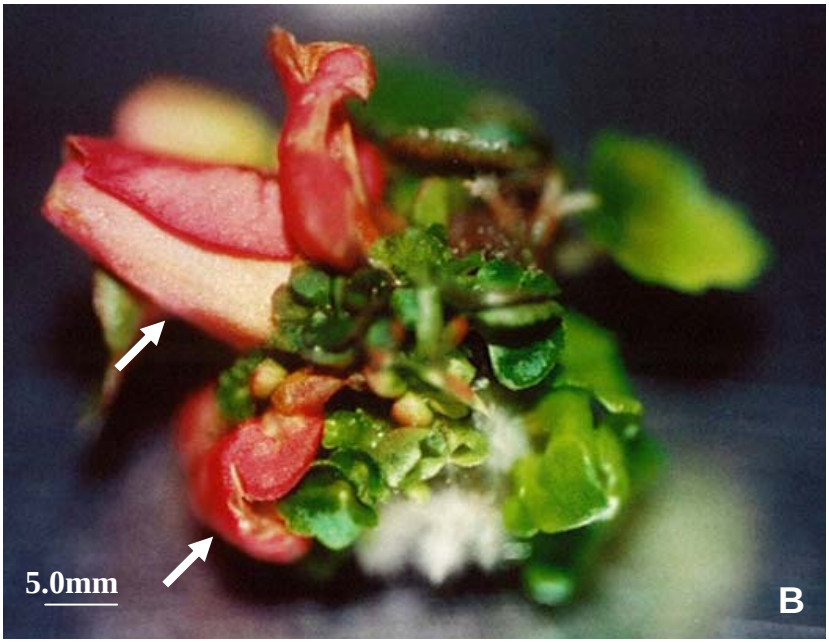


Plate 6.2: (A) (B) *In vitro* flowering induction from inflorescence explants cultured on MS medium supplemented with adenine, combinations of BAP and NAA and incubated for 8 weeks at 25 ± 1 ºC with 16 hours light and 8 hours dark.

Addition of both adenine and sucrose resulted in floral initiation and floral development after 4 weeks from inflorescence explants. The results showed that 40 mg/l adenine would increase the formation *in vitro* flowering bud. Thus, 40 mg/l adenine was selected for *in vitro* flowering induction in Begonia (Table 6.1).

6.3.3 The Effect of Different Concentrations of Sucrose on *In Vitro* Flowering

The optimum number of flowers produced per explant increased with increasing sucrose concentration up to 4.0% (w/v). Among the different concentrations of sucrose tested in this study (1.0-5.0%), 4.0% (w/v) sucrose induced the highest numbers of flowers (13.75%) but the results were not significantly different as compared with other concentrations (Table 6.2). Higher concentrations of sucrose (5.0%) produced the highest percentage of vegetative shoots and also caused the flowers to abscise while lower concentrations of sucrose (1.0-2.0%) did not induce *in vitro* flower (Table 6.2).

6.3.4 The Effect of Different Concentrations of Adenine on *In Vitro* Flowering

Table 6.3 shows the effect of adenine which was added exogenously at various concentrations ranging from 2.0-10.0% (w/v) to MS media in the presence of 1.0 mg/l BAP and 1.0 mg/l NAA. Multiple vegetative shoot formation up to 38.50% was observed from the inflorescence explants within 8 weeks of incubation. The same explant managed to produce flower buds (3.60%) and also formed roots on the same media. Some roots were observed in aerial position and did not touch the medium.

Table 6.2: The effect of different sucrose concentration on *in vitro* flowering induction of inflorescence and peduncle explants of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red* cultured on the MS media supplemented with 1.0 mg/l BAP,1.0 mg/l NAA and 40 mg/l adenine at 25 ± 1 ºC with 16 hours light and 8 hours dark.

SUCROSE % (w/v)	Observations		*Explants with green shoots (vegetative) (%) (Mean ± SE)		*Explants with red shoots (reproductive) (%) (Mean ± SE)	
	Inflorescence	Peduncle	Inflorescence	Peduncle	Inflorescence	Peduncle
0	Browning	Browning	**n.r	**n.r.	**n.r.	**n.r.
1	Vegetative green shoots and roots	Vegetative green shoots and roots	11.50±6.09 $_{c}$	26.00±5.03 $_{c}$	**n.r.	**n.r.
2	Vegetative green shoots and roots	Vegetative green shoots and roots	31.00±6.94 $_{b}$	30.50±8.87 $_{bc}$	**n.r.	**n.r.
3	Vegetative green, red shoots and roots	Vegetative green, red shoots and roots	40.50±9.98 $_{a}$	48.00±5.72 $_{a}$	8.50±2.35 $_{a}$	7.75±2.55 $_{a}$
4	Vegetative brownish shoots and roots	Vegetative brownish shoots and roots	36.50±11.25 $_{a}$	37.00±3.18 $_{b}$	6.55±2.22 $_{a}$	11.00±6.41 $_{a}$
5	Vegetative reddish shoots and roots	Vegetative brownish shoots and roots	31.75±8.15 $_{b}$	45.00±8.43 $_{a}$	6.55.00±2.22 $_{a}$	13.75±5.59 $_{a}$

* Mean values followed by the same letters within a column are not significantly different at the 0.05 level according to LSD test.

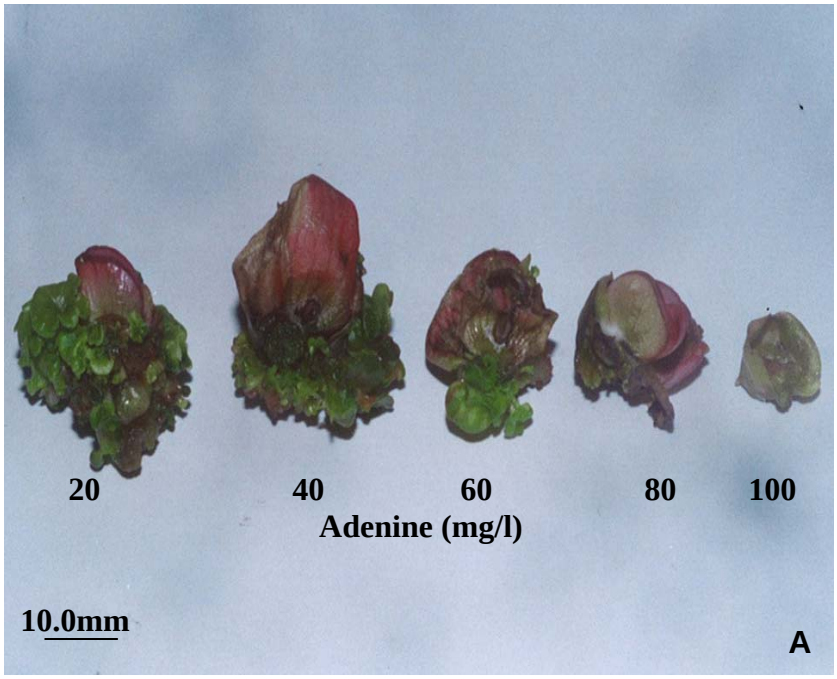
**n.r. = No response

Table 6.3: The effect of different adenine concentrations on *in vitro* flowering induction of inflorescence and peduncle explants of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red* cultured on the MS media supplemented with 1.0 mg/l BAP, 1.0 mg/l NAA and 3.0% sucrose at 25 ± 1 ºC with 16 hours light and 8 hours dark.

ADENINE (mg/l)	Observations		*Explants with green shoots (vegetative) (%) (Mean ± SE)		*Explants with red shoots (reproductive) (%) (Mean ± SE)	
	Inflorescence	Peduncle	Inflorescence	Peduncle	Inflorescence	Peduncle
20	Multiple green shoots, flower buds and roots	Multiple green shoots, flower buds and roots	42.00±6.16 a	40.00±10.26 a	2.65±1.42 a	6.70±2.00
40	Multiple green shoots, flower buds and roots	Multiple green shoots, flower buds and roots	38.50±1.09 a	24.00±9.40 b	3.60±0.82 a	**n.r.
60	Multiple green shoots, flower buds and roots	Multiple shoots and roots	15.00±5.13 b	8.40±3.79 c	2.05±1.35 a	**n.r.
80	Multiple brownish shoots and roots	Multiple brownish shoots and roots	10.25±3.79 b	5.00±0.00 c	**n.r.	**n.r.
100	Multiple reddish shoots and roots	Multiple brownish shoots and roots	5.00±0.00 c	**n.r.	**n.r.	**n.r.

* Mean values followed by the same letters within a column are not significantly different at the 0.05 level according to LSD test.

**n.r. = No response



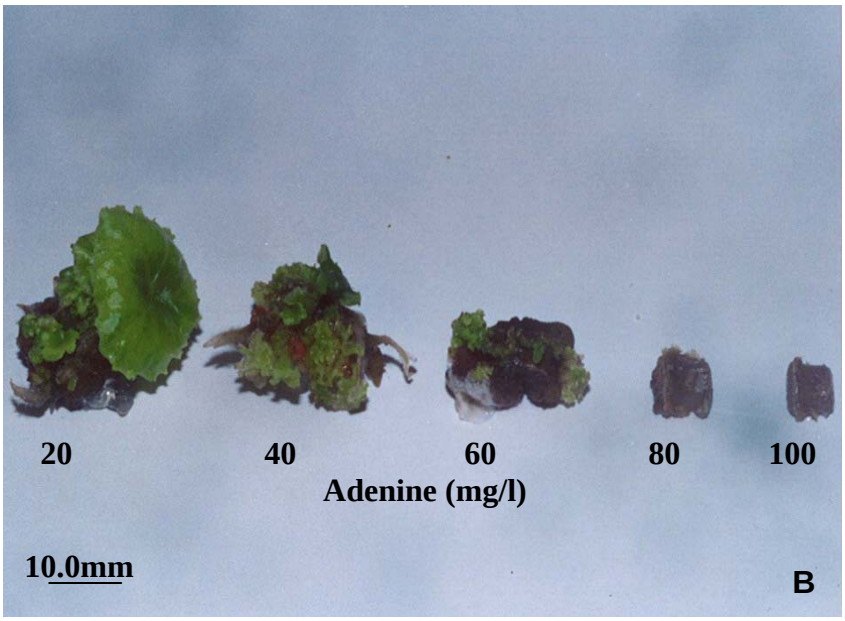


Plate 6.3: The effect of different concentrations of adenine on the induction of *in vitro* flowering from inflorescence (A) and peduncle explants (B) of *Begonia* x *hiemalis* Fotsch. after 8 weeks incubation.

6.3.5 The Effect of Different Dark Incubation Periods on *In Vitro* Flowering

Different types of photoperiods were tested in order to find out whether *in vitro* flowering would be produced (Table 6.4). When inflorescence and peduncle explants were cultured for 8 weeks on optimum media (MS supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA), a high percentage of vegetative buds was formed (73.00%) from inflorescence explants after 21 days incubated in the dark. The percentage of reproductive buds (18.50%) was produced from inflorescence explants after 12 days of incubation period. The results from Table 6.4 showed that by culturing the explants for a short period of darkness especially for 12 days was beneficial to *in vitro* flowering induction of inflorescence explants (18.50%). Incubation of explants in the dark resulted in the production of a very low percentage of reproductive buds but a high percentage of vegetative buds.

Table 6.4: The effect of different dark incubation period on *in vitro* flowering induction of inflorescence and peduncle explants of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red* cultured on MS media supplemented with 1.0 mg/l BAP, 1.0 mg/l NAA, 3.0% sucrose and 40 mg/l adenine at 25 ± 1 ºC with 16 hours light and 8 hours dark.

DARK INCUBATION PERIOD (days)	Observations		*Explants with green shoots (vegetative)(%) (Mean ± SE)		*Explants with flower buds (reproductive)(%) (Mean ± SE)	
	Inflorescence	Peduncle	Inflorescence	Peduncle	Inflorescence	Peduncle
2	Multiple green shoots and roots	Multiple green shoots and roots	53.00±9.49 $_{c}$	10.00±0.00 $_{d}$	14.00±5.16 $_{b}$	**n.r
4	Multiple green shoots and roots	Multiple green shoots and roots	49.00±11.93 $_{c,d}$	37.00±3.49 $_{c}$	8.50±2.41 $_{c}$	7.50±2.63 $_{b}$
6	Multiple shoots and roots	Multiple shoots and roots	52.00±10.32 $_{c}$	46.00±5.16 $_{a}$	16.0±05.16	4.60±1.26 $_{c}$
8	Multiple brownish shoots and roots	Multiple brownish shoots and roots	53.00±13.37 $_{c}$	35.00±8.49 $_{c}$	7.00±2.58 $_{c}$	13.5±5.79 $_{a}$
10	Multiple reddish shoots and roots	Multiple brownish shoots and roots	50.00±14.91 $_{c}$	42.00±4.21 $_{b}$	11.00±3.16	1.00±0.66 $_{d}$
12	Red leaves	Yellowish callus	44.00±8.43 $_{d}$	40.00±0.00 $_{b,c}$	18.50±2.41 $_{a}$	4.50±1.58 $_{c}$
14	Red leaves and yellowish callus	Pink and yellowish callus	53.00±10.29 $_{c}$	33.00±8.23 $_{c}$	15.00±5.27 $_{b\,a}$	13.00±4.83 $_{a}$
21	Read leaves, pink, yellowish and white callus	Pink, yellowish and white callus	73.00±10.97 $_{a}$	31.00±5.68	13.50±5.80	8.00±2.58 $_{b}$
28	Red leaves, pink and white callus	Pink and yellowish callus	62.00±11.35 $_{b}$	34.00±5.16	16.00±5.16 $_{a}$	6.57±1.37 $_{b}$

* Mean values followed by the same letters within a column are not significantly different at the 0.05 level according to LSD test. **n.r. = no response.

6.3.6 *In Vitro* and *Ex Vitro* Flowering of The Plantlets Obtained from Various Experiments

Plate 6.4 shows *in vitro* flowering of Begonia was obtained from the *in vitro* plantlets after 4 months cultured on MS medium devoid of plant growth regulators and supplemented with 40 mg/l adenine. *In vitro* developed flowers were morphologically similar to the *in viv*o flowers except for their smaller sizes and the petals were light pink colour.

Plantlets that have been acclimatized from different *in vitro* treatments on MS medium supplemented with 0.5 mg/l GA_3 showed continuous growth whereby the plants attained their maturity with the increasing of their height. The results showed that the inflorescence emerged from the shoot tips of the plantlets after 5 months of undergoing acclimatization process (Table 6.5, Plate 6.5 and 6.6). The complete cycle, from *in vitro* induction of plantlets to the flowering of regenerated potted plants, took 6-7 months i.e. less than with the conventional methods. The results showed that a high frequency of true-to-type flowering exhibited from the inflorescences that were cultured *in vitro*. Based on the present observation, the inflorescence emerged from the shoot tips of the plantlets. Almost 100.00% of the plantlets managed to produce red flowers which were similar with flowers that were obtained from acclimatized plants which were derived from various experiments including regeneration protocol, direct somatic embryos production, indirect somatic embryos and synthetic seed formation (Table 6.5). Flowering was very homogenous and no variant phenotype was observed within regenerated plants. Generally, two kinds of floral induction were observed among the flowering samples including normal (Plate 6.5) and abnormal floral formation (Plate 6.4). *In vitro* developed flowers were similar to the *in viv*o flowers except for their smaller sizes.

Table 6.5: *In vitro* and *ex vitro* flowering of *Begonia* x *hiemalis* Fotsch. on different types of substrates derived from different experiments.

Substrates	Types of experiments	Observations
Top soil 1	Organogenesis 12 Light, 12 dark	Flowers
Top soil 1	Organogenesis 16 Light, 8 dark	No flower
Top soil 2	Organogenesis	*Ex vitro* red flowers
Sphagnum	Organogenesis	*Ex vitro* red flowers
Vermiculite	Organogenesis	*Ex vitro* red flowers
MS basal	Organogenesis	*In vitro* pink flower
Top soil 1	Direct somatic embryogenesis	*Ex vitro* red flowers
Top soil 1	Indirect somatic embryogenesis	*Ex vitro* red flowers
Top soil 1	Encapsulated micro shoots	*Ex vitro* red flowers
Top soil 1	Encapsulated somatic embryos	*Ex vitro* red flowers

Plate 6.4: The *in vitro* plantlets (A) produced flowers (B) after 4 months cultured on MS medium devoid of plant growth regulators and supplemented with 40 mg/l adenine.

Plate 6.5: The height of the plantlets during flowering (15.0 cm) and after being transferred to the top soil 1 in the greenhouse (A, B and C).

Plate 6.6: The *in vitro* plantlets successfully produced flowers (A and B) after 3 months being grown in the sphagnum substrate and after transferred to the greenhouse.

6.4 SUMMARY

1.0 The best explant source for *in vitro* flowering induction in Begonia was immature inflorescences.

2.0 The best induction media for *in vitro* flowering of Begonia was MS supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA, with 40 mg/l adenine.

3.0 The incubation of explants under different period of dark treatment did not give positive effect on *in vitro* flowering induction in Begonia.

4.0 The *in vitro* plantlets produced abnormal *in vitro* flowers after 4 months being subcultured onto MS supplemented with 40 mg/l adenine and devoid of plant growth regulators.

5.0 The regenerants derived from different experiments were successfully induced to produce flowers after 3 months (9 months starting from *in vitro* culture process until transferred to the greenhouse).

6.0 From the macromorphology studies such as colour and the size of *in vitro* flowers derived from tissue culture methods, it was found that the flowers of normal stock plants were similar with the *in vitro* flowering.

CHAPTER 7

ACCLIMATIZATION OF *Begonia* x *hiemalis* Fotsch.

7.1 EXPERIMENTAL AIMS

Acclimatization is not unique to micropropagation but has been used for years in conventional propagation (Preece and Sutter, 1991). Acclimatization is a very vital factor in plant tissue culture technique. Most species grown *in vitro* require an acclimatization process in order to ensure that sufficient number of plants survive and grown vigorously when transferred to soil (Hazarika, 2003). The process of acclimatization can begin while the plantlets are still growing *in vitro* (Preece and Sutter, 1991). Acclimatization process in tissue culture technique involves the adaptation of *in vitro* plantlets into natural habitat or normal environment after undergoing maturation period in the vials. The ability to adapt into new environment of in vitro plantlets when transferred to soil is crucial for the life cycle of regenerants.

The ultimate aim of the present chapter was to measure the survival rates of acclimatized or hardening plantlets derived from *in vitro* regeneration, somatic embryos induction, suspension culture, synthetic seed production and *in vitro* flowering. At the same time, different supporting media were identified for acclimatization purposes. The

optimum growths of the plantlets were also measured for each treatment and the growth percentage of the acclimatized plantlets were determined.

Apart from that, macromorphology and microscopic studies were also carried out to define the similarity or differences amongst the different types of plantlets. Macroscopic study was done by measuring the height of the plantlets, width and length of the leaves and any modification or variation occurred to the plantlets. Microscopic study was carried out by using scanning electron microscopy (SEM) to observe the ultra cellular structures regarding *in vitro* regeneration of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red*. *In vitro* plantlets produced from different types of treatments including from *in vitro* regeneration, somatic embryogenesis, suspension cultures, synthetic seeds and acclimatized plantlets were compared with the intact plants or the mother plants.

7.2 MATERIALS AND METHODS

7.2.1 Plant Materials and Acclimatization Conditions

Prior to acclimatization process, the containers containing *in vitro* plantlets derived from different types of experiments were opened. The plantlets must reach the height of about 4.0-5.0 cm before being transferred to soil or substrates. The well-rooted *in vitro* plantlets, which were normally at the age of 4-6 months old, were selected for this study. The roots were washed under running tap water to get rid of the agar and medium which may attract the microorganisms such as fungi, bacteria etc. The plantlets were soaked in antifungal solution such as benlate to prevent contamination. The acclimatized plantlets were grown in the black polyethylene bags or plastic containers or pots (measuring 20 x 25 cm), containing topsoil or substrates. The plantlets in the pots or polyethylene bags were covered with plastic bags, which had holes all over them to give some aeration to the plantlets. The plantlets were then transferred to the shaded greenhouse. Fertilizer (10N-22.7P-8.3K, 0.9 g/l) was applied once a week after the acclimatization process commenced.

7.2.2 The Effect of Different Types of Growing Substrates on Acclimatization

In order to determine the optimum growth of the *in vitro* plantlets, different types of substrates were used in this study. Four different types of substrates were applied

including topsoil 1, topsoil 2, sphagnum and vermiculite. Topsoil 1 is the normal conventional soil containing a mixture of 2 peat:1 soil:1 sand (by volume) fed with 10N-22.7P-8.3K fertilizer (0.9 g/l). Topsoil 2 is the soil derived from the pots belongs to donor plants from which the explants were obtained which containing a mixture of 8 coco peat: 1 perlite (expanded sand) and added up with fertilizers 10N-22.7P-8.3K fertilizer (0.9 g/l). Sphagnum was bought from local plant nursery in Sungai Buloh, Selangor. Vermiculite is a natural mineral that is formed by hydration of certain basaltic minerals and was obtained from Sigma-Aldrich Company. Sphagnum and vermiculite were also fed with 10N-22.7P-8.3K fertilizer (0.9 g/l). The plantlets were grown in a shaded greenhouse. Several parameters such as survival rate of the plantlets, plantlet height, leaf width and length were measured in each plantlet prior and after acclimatization process.

7.2.3 The Effect of Different Types of Plantlets Derived from Various Experiments

All plantlets regenerated from different treatments such as from *in vitro* regeneration, somatic embryos induction, suspension cultures process, synthetic seed production, and *in vitro* flowering were grown using the topsoil 1. The plantlets were also grown in a shaded greenhouse. Several parameters such as survival rate of the plantlets, plantlet height, leaf width and length were measured in each plantlet prior and after acclimatization process.

7.2.4 The Effect of Different Types of Environmental Cultural Factors

Begonia x *hiemalis* Fotsch. is a temperate plant which normally favours and easily survive in the temperate region. In this study, the plantlets were acclimatized under two different environmental factors and these factors normally control by relative humidity, different photoperiod and temperature. The acclimatized plantlets were maintained in the tissue culture room (Treatment 1) (under 25 ± 1 °C, 16 hours light, 8 hours dark) and the green house (Treatment 2) (29 ± 1 °C, 12 hours light, 12 hours dark) from two to twelve months of incubation period. The growth and survival rates of the regenerants from two different environmental conditions were then observed.

7.2.5 Measurement of Chlorophyll Content of *In Vivo*, *In Vitro* and Acclimatized Plants

The chlorophyll content of different ages of *Begonia* x *hiemalis* Fotsch. was measured by using spadmeter (SPAD = Soil Plant Animal Department of Minolta). The leaves of three different stages of plantlets including *in vivo* plant, *in vitro* plantlets and acclimatized plantlets were scanned by using spadmeter and the total *in situ* chlorophyll content were obtained from the machine.

7.2.6 Macromorphological Studies of *In Vivo, In Vitro* and Acclimatized Plants

Morphological structures of regenerated plants *in vitro* and from several regeneration systems were examined carefully. Morphology of leaf, stem and flower were observed and recorded. The differences that occurred between selected samples were characterized and compared with the normal intact plants.

7.2.7 Micro Morphological Studies of *In Vivo, In Vitro* and Acclimatized Plants

Microscopic studies of different types of plantlets obtained from different treatments were carried out by using scanning electron microscope (SEM) and microphotography microscope. The experiments were carried out to observe the changes that may occur in the regenerants. The specimens were dried prior to running the test in the vacuum system. Preparation of specimens can be categorized into several steps including air drying technique, low temperature technique and chemical bonding technique followed by critical point drying technique.

Air-drying technique was done in the normal room whereby the leaves samples from *in vivo, in vitro* and acclimated plants were placed in the room temperature. The specimen was washed with alcohol and sticked on using liquid carbon. This should be done simultaneously in order to avoid the carbon ink from dried up.

The leaves samples were treated with different types of chemicals. The sample was incubated in 30 ml of gluteraldehyde mixed with 30 ml of phosphate buffer for 1 hour at room temperature. Then, the sample was rinsed with phosphate buffer solution and distilled water in 1:1 mixture. The sample was incubated in Osmium (4%) and distilled water in 1:1 mixture for 14 hours at 4º C. The sample was rinsed with distilled water and fixed using the ethyl alcohol series (10% to 100%) for 15 min. each step, and followed by 3:1, 1:1 and 1:3 ethyl alcohol and acetone for 20 min. Then, the sample was incubated in Aseton (100%) for 20 min and this step was repeated four times. Lastly, the sample was sticked onto the specimen holder, plated and double-checked for conformation.

The sample was dried up in the liquid CO_2 for several times using CPD equipment to replace the acetone with carbon dioxide. The temperature was increased until liquid CO_2 changed to gas to avoid surface tension of the sample. Then, the specimen is ready to be plated with gold dust to stabilize the term and electricity of the sample. The sample was attached to special aluminum pin using conductive carbon cement. Then the pin was placed inside of SPI-Module Sputter Coaster chamber. The air of the chamber was placed with argon gas using a pump, which has been attached to the gold coater equipment. The sample was coated with gold particle for 60 seconds. Finally the specimen was observed using SEM (JEOL, JSM. 6400, Tokyo, Japan).

7.2.8 Statistical Analysis

All data and variables were statistically analyzed using SPSS statistical package version 11. Values are presented as mean ± SE. Mean percentage and Multiple Range Analysis were done on all data, using 95% LSD intervals method.

7.3 RESULTS

All plantlets regenerated from different treatments such as from *in vitro* regeneration, suspension cultures process, somatic embryos induction, synthetic seed production, and *in vitro* flowering adapted well to acclimatization process. Table 7.1 shows that most of the plantlets developed into healthy plants in the new planting media consisting of topsoil 1 (87.00%), topsoil 2 (8.00%), sphagnum (83.33%) and vermiculite (56.67%). The plant height increased rapidly during 6-9 months of transfer to the soil. The current investigation also showed that most of the plantlets managed to induce roots directly from *in vitro* regeneration. The presence of roots in the plantlets will be beneficial to the successful acclimatization process in *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red* and the results showed that root formation was optimum at 24-27 ºC. The length of roots did not vary significantly with the treatments.

Abnormal morphological structures were not found either from *in vitro* plantlets or regenerated plants. The observation was especially noted on the plant height and leaf width. The mean height and leaf width of the transplanted plants were 7.00 cm and 4.50 respectively, after 6 months being acclimatized. The results showed the increasing of growth rate after transferring them to the garden (Fig. 7.1).

From the present observation, one of the main problems encountered during the acclimatization process was the regenerants were very susceptible to many pathogenic

bacteria and fungi. This can be eliminated by watering the substrates of the regenerants rather than the leaf areas of the regenerants. Another method was to soak the plantlets in antifungal solution before transferring to pots or soil or garden.

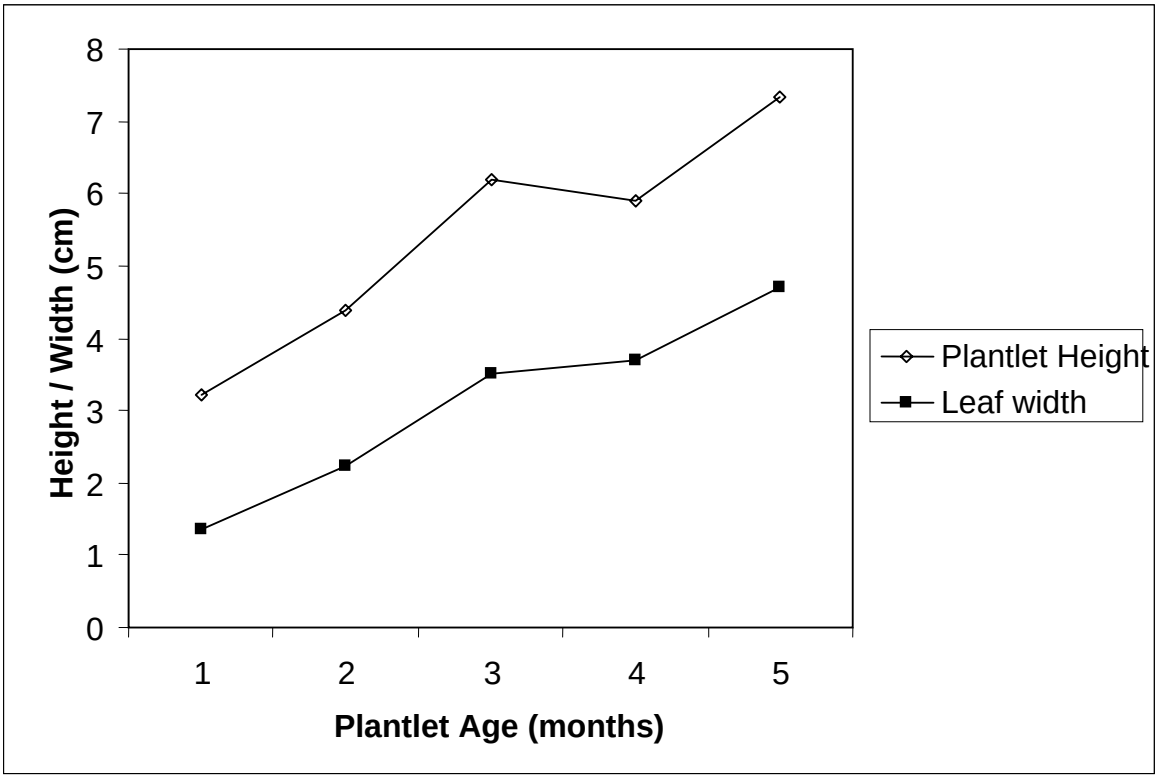


Figure 7.1: The growth rate of the acclimatized plantlets during hardening period on topsoil 1.

7.3.1 The Effect of Different Types of Growing Substrates on Acclimatization

Four different types of substrates were applied in this study including topsoil 1, topsoil 2, sphagnum and vermiculite. The results showed that the plantlets were well developed after being acclimatized to the 4 different types of substrates (Table 7.1, Plate 7.1). The survival rate of the regenerants from each substrate was high. However, the regenerants were well developed after being acclimatized on the topsoil 1 (87.00%), topsoil 2 (80.00%) and sphagnum (83.33%). Although the regenerants managed to grow well in vermiculite but the growth rate of the regenerants were lower (56.67%) compared with other substrates (80.00-87.00%).

Table 7.1: The growth rate of regenerants derived from *in vitro* regeneration process after being transferred to four different types of substrates in the greenhouse for 6 months.

Acclimatized plantlets from different substrates	Survival rates of the plantlets (% ± SE)
Top soil 1	87.00 ± 0.70 $_a$
Top soil 2	80.00 ± 0.08 $_a$
Sphagnum	83.33 ± 0.06 $_a$
Vermiculite	56.67 ± 1.20 $_b$

* Mean values followed by the same letters within a column are not significantly different at the 0.05 level according to LSD test.

Plate 7.1: Comparison of the different types of substrates used during acclimatization process. (A) Topsoil 1, (B) Topsoil 2, (C) Sphagnum and (D) Vermiculite.

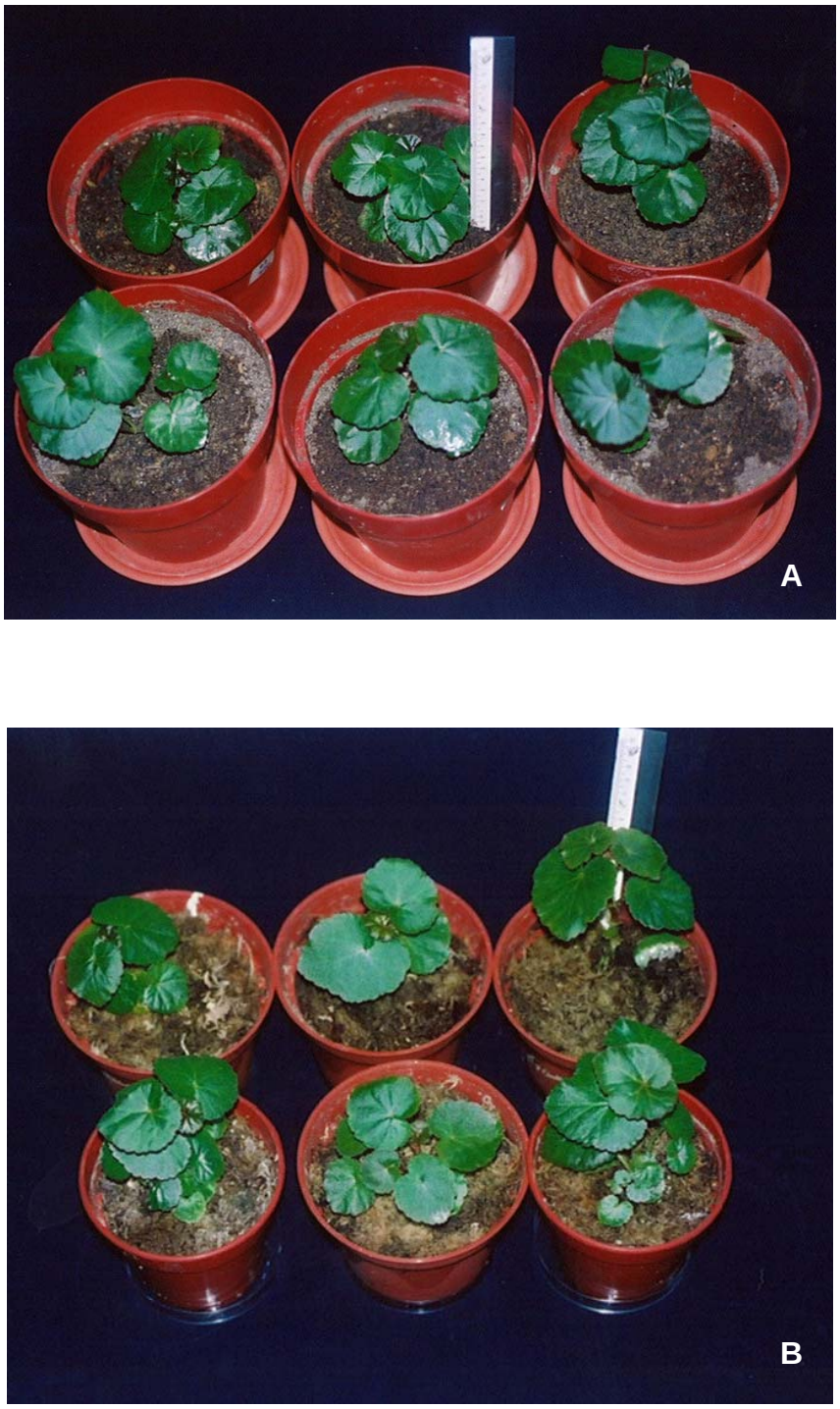


Plate 7.2: **The plantlets were successfully acclimatized on the different substrates (A) topsoil 1 and (B) sphagnum for 6 months in the greenhouse.**

7.3.2 The Effect of Different Types of Plantlets Derived from Various Experiments

The survival rates of most of the regenerants derived from different types of *in vitro* process treatments i.e. from *in vitro* regeneration, suspension cultures process, somatic embryos induction, synthetic seed production, and *in vitro* flowering were quite high. Plants acclimatized using topsoil media in the green house had survival rate of more than 80.00% except for the regenerants which were germinated from the synthetic seeds production (36.67% - 56.67%).

Table 7.2: The survival rates of regenerants derived from different types of *in vitro* process acclimatized on topsoil 1 in the greenhouse.

Acclimatized plantlets from different treatments	Survival rates of the plantlets (% ± SE)
Regeneration process	
1. BAP + NAA	80.00 ± 0.08 $_{c}$
2. TDZ	80.00 ± 0.08 $_{c}$
Suspension cultures	87.00 ± 0.70 $_{a}$
Somatic embryos	
1. Direct somatic embryogenesis	83.33 ± 0.06 $_{b}$
2. Indirect somatic embryogenesis	80.00 ± 0.08 $_{c}$
Synthetic seeds derived from micro shoots	56.67 ± 1.20 $_{d}$
Synthetic seeds derived from somatic embryos	36.67 ± 1.18 $_{e}$
In vitro flowering	80.00 ± 0.08 $_{c}$

* Mean values followed by the same letters within a column are not significantly different at the 0.05 level according to LSD test.

7.3.3 The Effect of Different Types of Environmental Factors on Acclimatization

The growth and survival rates of the regenerants acclimatized under two different environments were examined in this experiment. The regenerants which were kept in the tissue culture room (25 ± 1 °C, 16 hours light, 8 hours dark) grew more vigorously as compared with the regenerants grown in the green house (Plates 7.3 and 7.4). However, the regenerants from Treatment 1 could not produce flowers after 12 months undergoing acclimatization period, whereby the regenerants from Treatment 2 managed to produce flowers after 6 months of acclimatization process (Plate 7.3). This might be due to the response of the regenerants to the different types of environmental factors such as photoperiod effect, temperature and relative humidity.

7.3.4 Measurement of Chlorophyll Content

The chlorophyll content from young and mature leaves of *in vivo* plants, *in vitro* plantlets and acclimatized plantlets were determined to compare the chlorophyll content between treatments. The results showed that there is no significant difference in the chlorophyll content between the young and mature leaves of *in vivo* plants, *in vitro* plantlets and acclimatized plantlets (Fig. 7.2).

Plate 7.3: Comparison of the heights of the plantlets after 9 months transferred onto topsoil 1 and being acclimatized on different environment in the green house (A) and culture room at 25 ± 1°C (B).

Plate 7.4: Twelve-month-old plantlet during hardening process in the culture room at 25 ± 1°C.

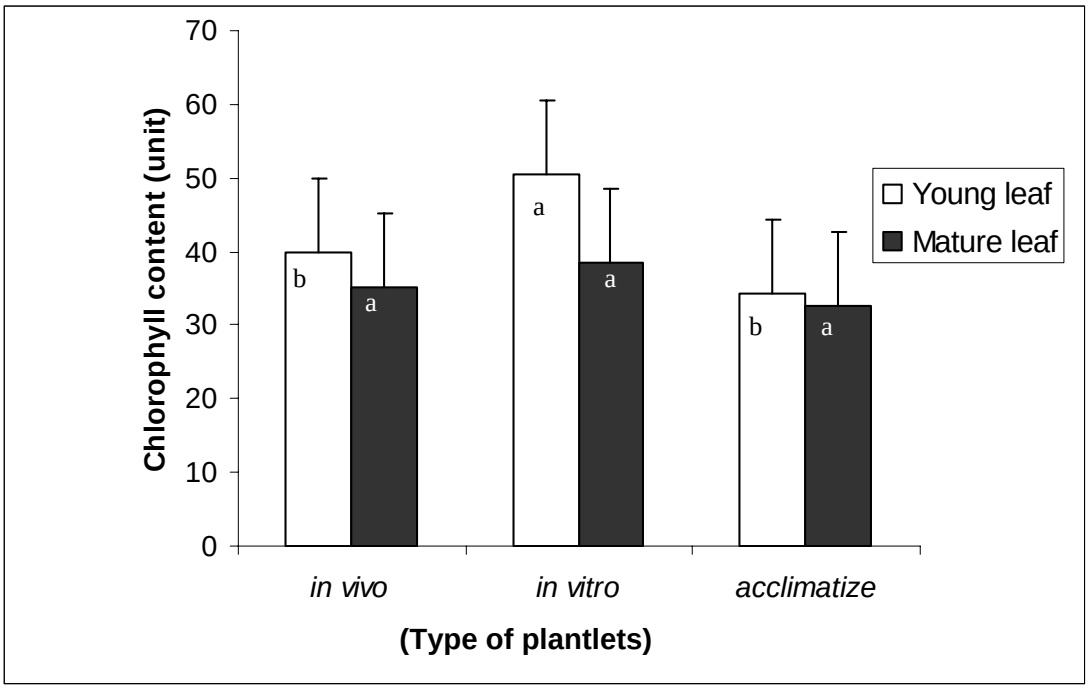


Figure 7.2: Comparison of chlorophyll content of *in vitro* and acclimatized plantlets with *in vivo* plants.

7.3.5 Macromorphological Observations

Based on the present investigation, there was no morphological aberration observed either in *in vivo* plants or *in vitro* plantlets and also acclimatized regenerants of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red*. The sizes of the leaves ranged from under 2.0 cm to over 12.00 cm depending on the age of the plants. The leaf surfaces were glabrous. The stems were fleshy and the leaves were all lope-sided. The inflorescences were usually axillary with unisexual and zigomorphic flowers and the flowers were red in colour. Flowers were characteristically monoecious with separate male and female flowers, and the pistillate flowers have inferior ovaries (Plate 6.5, Chapter 6).

7.3.6 Micromorphological Observations

Scanning electron microscopy (SEM) research can reveal detail ultrastructures of *in vitro* plants derived from different treatments. Different types of plantlets were compared with the original plants. The results obtained showed the morphological structures of the leaf surfaces and the differences between treatments. Plate 7.5 (A-F) shows the scanning electron micrographs of the abaxial surface structure of the leaf from different treatments. The leaf surface consisted of epidermis cells, guard cells, stomata and trichomes.

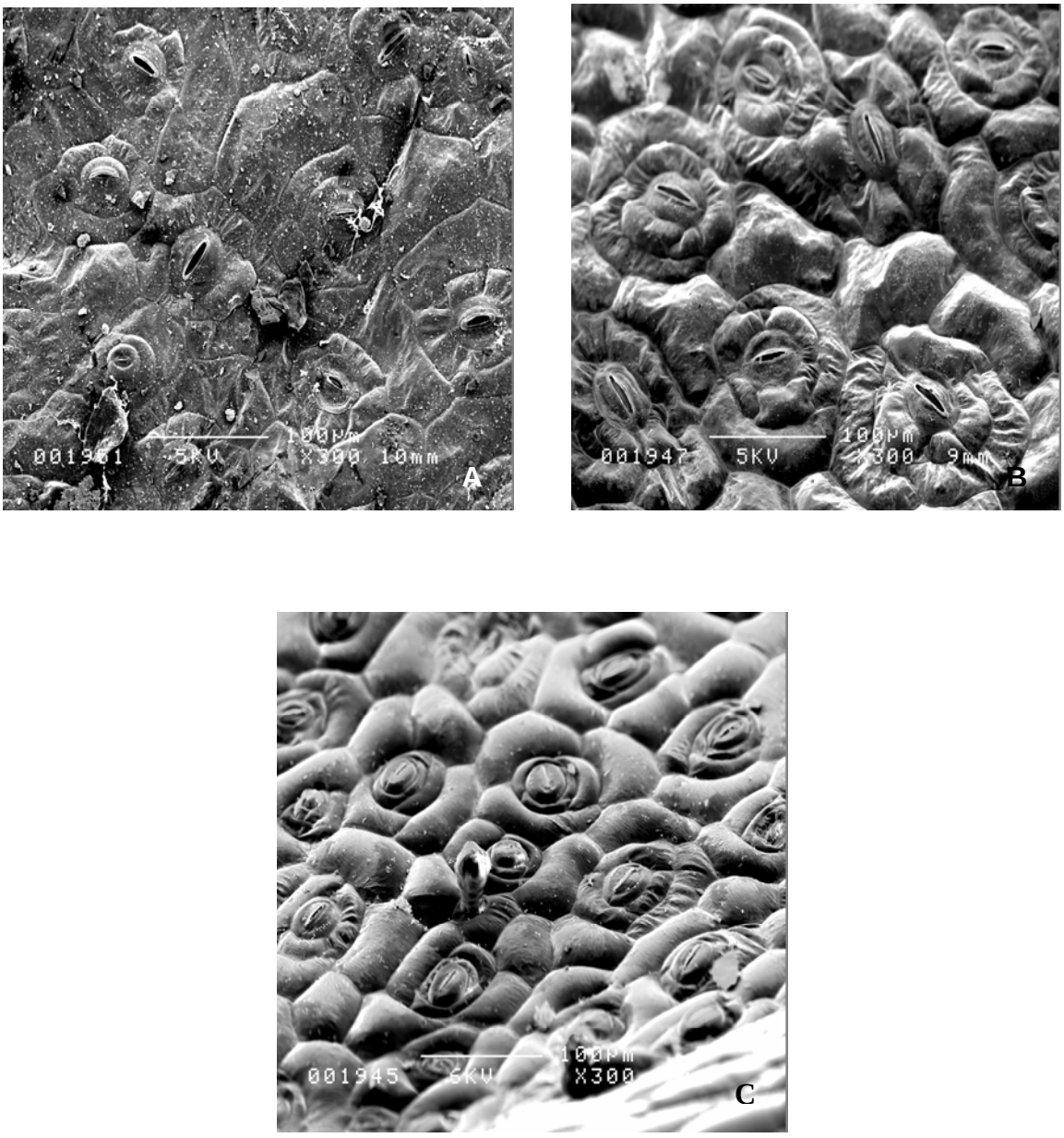


Plate 7.5a: Scanning electron micrographs of the leaf surfaces from different pathways of regeneration of *Begonia* x *hiemalis* Fotsch. var. *Swabenland Red*. (A) Intact plant. (B) I*n vitro* regeneration from organogenesis. (C) Acclimatized plantlet.

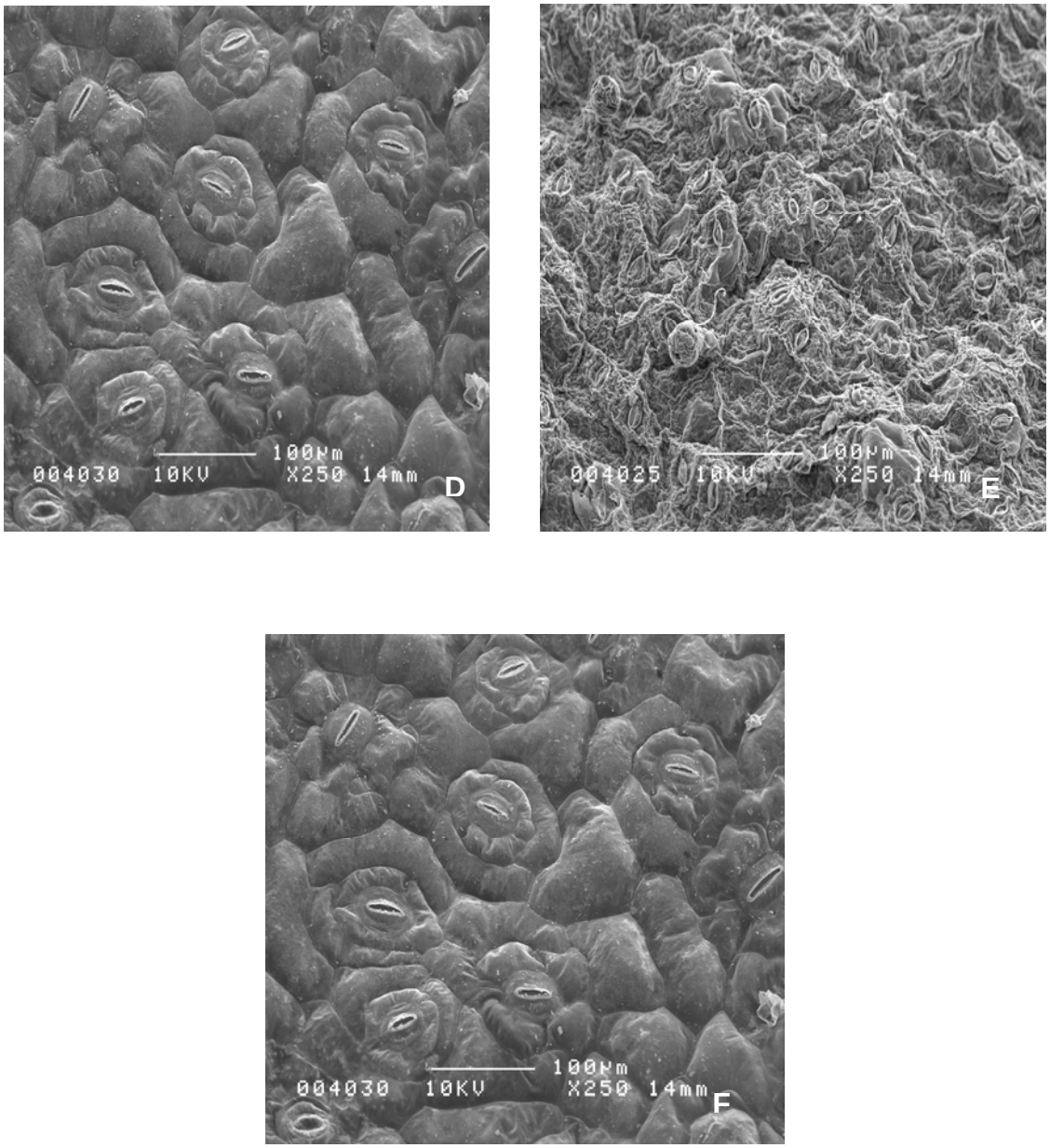
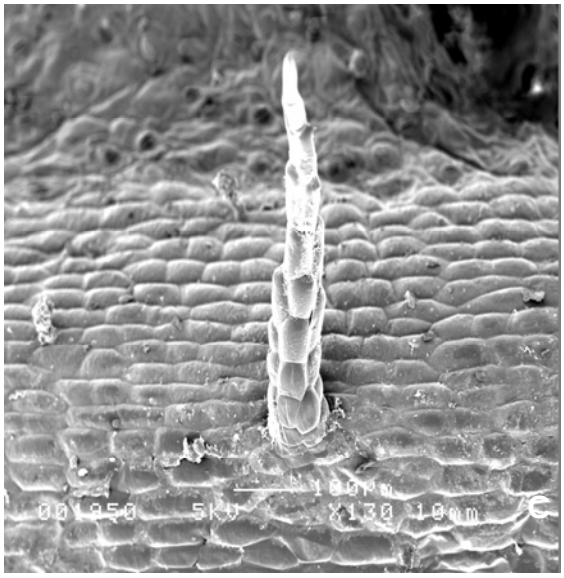
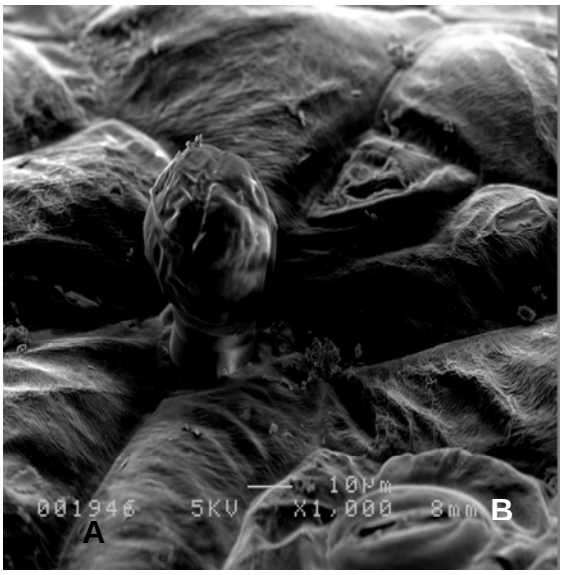


Plate 7.5b: Scanning electron micrographs of the leaf surfaces from different pathways of regeneration of *Begonia* x *hiemalis* Fotsch. var. *Swabenland Red*. *In vitro* plantlet derived from (D) suspension cultures, (E) somatic embryogenesis process and (F) synthetic seed production. (Bar represents 100 µm)





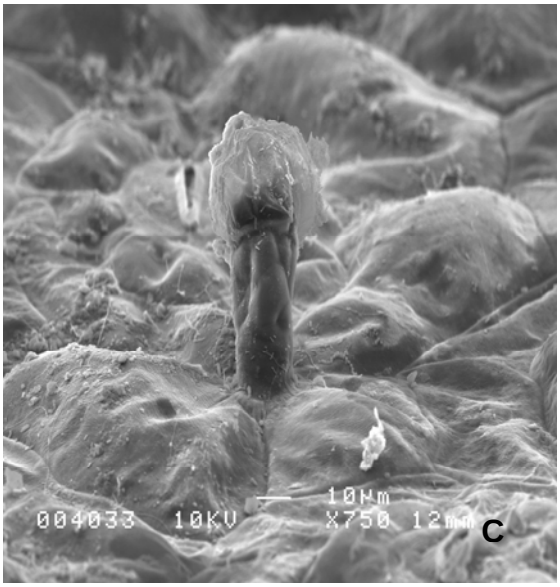
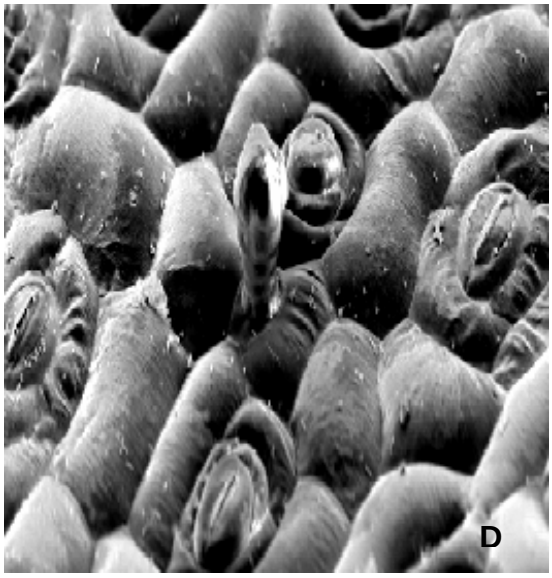
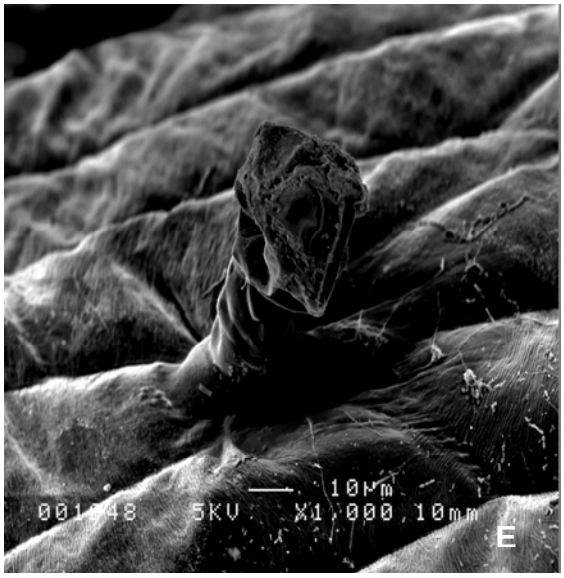


Plate 7.6a: Scanning electron micrographs of trichomes from different plantlets. The trichomes formed on the surface of the abaxial leaves from intact plant (A), *in vitro* plantlet (B), acclimatized plantlet (C). Bar in each figure represents 10 µm except for intact plant represents 100 µm.





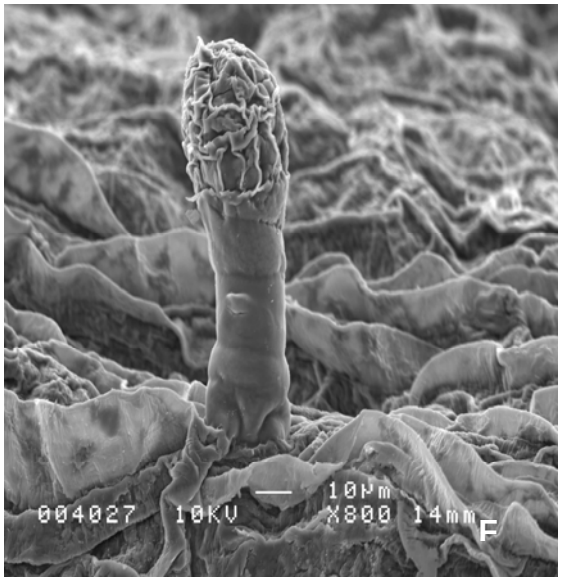


Plate 7.6b: Scanning electron micrographs of trichomes from different plantlets. The trichomes formed on the surface of the abaxial leaves from suspension cultures (D), embryoid (E) and synthetic seed (F). (Bar in each figure represents 10 µm)

7.4 SUMMARY

1. Most of the Begonia plants regenerated from *in vitro* system adapted well when transferred to soil during hardening process.

2. Most of the substrates employed during acclimatization process supported plant growth such as topsoil 1, topsoil 2 and sphagnum.

3. About 80.00% of the regenerated plantlets derived from different treatments managed to survive and grew into normal plants when transferred to topsoil 1.

4. Most of the plantlets started to produce flowers after 9 months being transferred to the green house except for the plants acclimatized in the culture room.

5. The transplanted plants that were acclimatized at 25 ± 1 ºC with 16-hours photoperiod for 12 months did not produce flower but grew vigorously.

6. Generally, the morphological structures of the *in vitro* plantlets were similar when compared with the intact plants of Begonia, whereby the leaves were dark green in colour, thick, shiny with elliptic and ovate structures.

7. The chlorophyll content of intact and transplanted plants is not significantly difference between the *in vivo*, *in vitro,* and acclimatized plantlets.

8. Abnormal micromorphological structures were not found in any regeneration pathways i.e. direct, indirect somatic embryogenesis, suspension cultures etc. For micromorphological studies, the parameters used were stomata and trichomes, by comparing the morphology of stomata and trichome between the different regeneration systems.

9. Based on these experiments, multiplication of Begonia from various tissue culture pathways (organogenesis, somatic embryogenesis, cell suspension culture) proved to produce almost similar offsprings / plants which can be beneficial for commercialization of Begonia.

CHAPTER 8

8.0 DISCUSSION

Plant tissue culture technique is an alternative method for mass propagation or cloning of Begonia plants and also to overcome the problems occurring in the conventional propagation methods (Rout *et al.*, 2006). In the current study, plant tissue culture of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red* was successfully carried out which involving the process of *in vitro* regeneration, callus induction, initiation of suspension cultures, the process of indirect and direct somatic embryos induction, formation, storage period and germination capability of the synthetic seeds, *in vitro* and *ex vitro* flowering induction and finally acclimatization or hardening process. Various factors such as different combinations and concentrations of growth regulators (BAP, NAA, TDZ, IAA, kinetin and etc.), different concentrations of sucrose, different pH levels, photoperiod and other additives such as coconut water, induction of multiple shoots through suspension cultures were analyzed in detail for this species.

Research which was done by Takayama and Misawa (1982) showed that Begonia propagated through in *vitro* technique did not reveal any phenotypical abnormality and managed to grow well and produce flowers even in mid-summer, while the plants propagated by stem cutting were easily damaged by fungal and bacteria attacks. Thus, tissue culture of Begonia (*Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red*) was done to produce disease-free plants and mass-propagate the Begonia for the purpose of economical and ornamental values. The main aim of these studies was to develop a new improve

protocol mainly for *in vitro* regeneration procedure, callus induction, suspension culture, synthetic seeds production, somatic embryogenesis induction, *in vitro* flowering, acclimatization process and to establish the micropropagation process in *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red.* Some previous studies (Ringe and Nitsch, 1968; Roest *et al*., 1981; Cassells and Morrish, 1985; Pierik and Tetteroo, 1987; Ringe and Nitsch, 1968; Castillo and Smith, 1997; Samyn *et al*., 1984 and Takayama and Misawa, 1982) were done on Begonia. This will be beneficial for the clonal production and development of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red* through various methods in plant tissue culture procedures for commercial purposes.

Regeneration via adventitious organogenesis in *in vitro* cultures of many species is associated with a high frequency of off-types (Cassells and Morrish, 1987b). *Begonia* x *hiemalis* Fotsch. is commercially propagated by leaf and top cuttings (Appelgren, 1976), implying high genome stability, also stable *in vitro*, where adventitious regenerants show little deviation from parent types (Cassells and Morrish, 1985). Thus, the *in vitro* regeneration of *Begonia* x *hiemalis* Fotsch. was investigated using various internal and external cultural factors such as different types of explants, different positions of explants, different types of plant growth regulators, different concentrations of sucrose, different pH levels, photoperiod effect and other additives such as coconut water and adenine.

Among other factors, selection of appropriate donor tissue is the most critical aspect affecting the performance of micropropagation systems (Simmonds, 1992). In the present study, donor plants were maintained in the culture room, with temperature of 25 ± 1°C and

16 hours light 8 hours dark, to maintain the quality of the plants and also protect the plants from being infected by any plant diseases. Based on previous research, it was found that the effects of temperature and photoperiod on the physiological status of the donor tissues could also affect subsequent *in vitro* responses (Cassells, 1979; Simmonds, 1992). The photoperiod does not directly influence the explant competence, but regenerative capacity is affected by the changing status of the donor plants in response to photoperiodic treatment (Simmonds, 1992).

Murashige and Skoog (1962) (MS) basal medium was used widely throughout the experiments and the explants were incubated at 25 ± 1°C with 16 hours light 8 hours dark, to induce the optimum growth quality of the explants. The observations were made within 8 weeks of culture period.

Prior to the identification of the optimum organogenesis process in Begonia, a preliminary study was carried out to examine the effect of different positions of explants on *in vitro* regeneration. Four different explants including leaves, peduncles, petioles and stems were cultured with horizontal and vertical position whereas the leaf explants were also cultured in upside down position onto MS media devoid of plant growth regulators. The results revealed that the effect of different position of explants gave a positive impact on the explants response on organogenesis process (Figure 2.1). The observations showed that the leaf explants which were placed on the horizontal position (abaxial surface down), with the physiological base on the medium gave significantly higher percentage of organogenesis (27.00%) as compared with another two different positions of explants i.e.

vertical (15.00%) and upside down position (16.00%). Apart from that, the results also showed that the peduncles, petioles and stems that were placed on vertical position showed high percentage of regeneration (21.00%, 23.00% and 22.00%) as compared with the horizontal position (16.00%, 17.00% and 18.00%). Thus, the present findings have clearly demonstrated that leaf explants gave the best organogenesis response as compared with other explants in the MS medium devoid of plant growth regulators.

From this study, four different intact explants of selected Begonia gave a similar response on *in vitro* micro shoots formation after being cultured for 8 weeks onto MS media containing various concentrations of BAP (0.1-5.0 mg/l) and NAA (0.1-3.0 mg/l). Depending on the medium, prior to the formation of shoot buds, the cut ends of explants swelled and then direct vegetative buds appeared as small green protuberances without any intervening callus phase. The morphological observations also showed that the high percentage of *in vitro* regeneration could be obtained especially from leaf (68.67 %) and petiole explants (66.00%). Even though micro shoots were successfully produced from peduncle (51.00%) and stem explants (51.00%) but the percentage was less as compared with leaf and petiole explants. Appelgren (1976) identified that peduncle segments of *Begonia* x *hiemalis* cv. *Schwabenland* was very dependent on the growth regulators concentration in the medium. Sunpui and Kanchanapoom (2002) had also examined the effect of different types of explants such as leaf, petiole and stem explants on the percentage of shoots formation in African violet. The results showed that the most responsive explants was petiole and then followed by leaf and stem explants. Their results also revealed that direct *in vitro* plant regeneration of African violet through tissue culture of leaf and petiole explants could be obtained easily.

Other experimental work in the present study which was undertaken concurrently includes using TDZ, cytokinins and auxins both singly and in combinations at low to very high levels on a variety of explants sources. Exposure to growth regulators in these trials ran for four, eight, twelve and sixteen weeks. An increase in shoot formation was observed in each explant depending on different combinations of plant growth regulators. Micro shoots were obtained on MS medium containing BAP (0.1-5.0 mg/l) and NAA (0.1-3.0 mg/l). After the optimization of BAP and NAA concentrations, an experiment was conducted to determine the effect of TDZ on regeneration and shoot induction from peduncle and stem explants. Following successful responses of explants different ranges of cytokinin and auxin combinations were investigated.

The findings showed that lower levels of BAP (0.1 mg/l) and NAA (0.1 mg/l) induced relatively fewer shoot buds (11.83-33.33%), but these developed rapidly into normal shoots. In contrast, MS media containing higher levels of BAP (5.0 mg/l) and NAA (1.0 g/l) had more stunted vegetative shoot buds but only a few developed into shoots. Fonnesbech (1974a) also claimed that lower concentrations of BA (0.001 mg/l) yielded no shoots, higher concentrations (3.0 mg/l) promoted shoot formation, but the shoots were abnormal with malformed leaves in *Begonia* x *cheimantha* petiole segments grown *in vitro*. Fonnesbech (1974a) also found that lower concentrations of NAA (0.001 mg/l) resulted in poorer survival rate and no roots, with higher concentrations of NAA (1.0 mg/l) many roots developed but these were thickened and their longitudinal growth inhibited. From this study, 1.0 mg/l BAP and 1.0 mg/l NAA was found to be the best combination of

growth regulators to be supplemented to MS medium in obtaining optimum regeneration in *Begonia* x *hiemalis* Fotsch cv. *Schwabenland Red*. Cassells and Morrish (1985) managed to induce buds from petiole of *Begonia rex* 'Lucille Closon' by using combinations of 0.1 mg/l BAP and 0.01 mg/l NAA. Fonnesbech (1974a) also managed to induce shoots and roots in *Begonia* x *cheimantha* petiole segments in modified White medium with 0.5-1.0 mg/l BA and 0.01 mg/l NAA. However, Appelgren (1976) proved that higher auxin (2.0 mg/l IBA) and lower cytokinin content (0.2 mg/l BA) induced shooting in peduncles of *Begonia* x *hiemalis* which is different from this research and also from the work of Cassells and Morrish, 1985; 1987; Fonnesbech, 1974a). Nakano *et al.* (1999) also managed to obtain elongated shoots of *Begonia* x *tuberhybrida* Voss in 0.1 mg/l NAA and 0.1 mg/l BA. By using shake-culture in a liquid medium containing NAA and BAP, the elongated shoots were most efficiently obtained (Nakano *et al.*, 1999), inducing proliferation and development of propagules (Peck and Cumming, 1984) and enhancing the micropropagation profile (Simmonds and Werry, 1987) in Begonia. This was also proven in this study.

A lot of work had been done to study the effect of sucrose and glucose concentrations on the growth of Begonia (Takayama and Misawa, 1981; 1982; Pierik and Tetteroo, 1987). Sucrose is the most common carbon source used in plant cell, tissue and organ culture media (Hazarika, 2003). In the present study, the highest percentage of shoots (70.94%) was obtained from peduncle explants cultured in MS media supplemented with 4.0% sucrose, then followed by 3.0% sucrose (65.00%). The stem explants also produced shoots in 4.0% of sucrose (66.88%) and in 3.0% sucrose (64.00%). The percentage of shoots formation decreased in the media with 5.0% sucrose. Takayama and Misawa (1982) have

found that with 3.0% sucrose, the buds were scarce in a ¼ MS liquid medium, and cultures appeared to be root cultures in *Begonia* x *hiemalis* Fotsch cv. *Schwabenland Red*. Later in 1982, Takayama and Misawa found that formation of buds at the base of the plantlets in 3.0% sucrose was superior to that in 1.0% sucrose medium. However, Pierik and Tetteroo (1987) studied the effect of glucose in shoot development of *Begonia venosa* and they found that the shoot development was promoted and leaf expansion occurred at low glucose levels (0.5-1.5%). Bhojwani and Razdan (1996) also suggested that the most common used carbon source is sucrose, at a concentration of 2.0-5.0%, and glucose and fructose are also known to support good growth of some tissues.

In the current investigation, other than the effect of different sucrose content, different pH media were also tested to obtain the best pH for the induction of *in vitro* regeneration in Begonia. The highest percentage of micro shoots (62.50% and 66.25%) was obtained from stem explants which were cultured on MS media with pH 4.3 and 5.8, respectively (Table 2.6). Skirvin *et al.* (1986) also suggested that the direction and extent of the pH change might be influenced by the optimum pH of parent plants *in vivo.* Pierik (1987) also predicted that a pH in the range of 5.0-6.5 is suitable for growth with a maximum at about 6.0, since low pH (lower than 4.5 and high pH (higher than 7.0) generally will stop the growth and development *in vitro*. In general, pH higher than 6.0 gives a fairly hard medium and a pH below 5.0 does not allow satisfactory gelling of the agar (Bhojwani and Razdan, 1996) that influence the availability of various mineral ions in the medium and their uptake by the plant tissue. Skirvin *et al.* (1986) also reported that there were significance differences between the initial pH levels and final pH levels following autoclaving, particularly in the pH range of 5.7 to 8.5.

In the present study, the highest percentage of micro shoots (70.94%) was achieved on MS media supplemented with 200 cm^3 of coconut water. Kovoor (1962) quoted by Pierik (1987), found that coconut milk contained a compound which was analogous to kinetin (a cytokinin). Addition of 100-150 cm^3 of coconut water was suggested into 1 liter of nutrient medium to supplement the growth ability of the explants.

Apart from achievement of *in vitro* regeneration in Begonia, callus induction was also investigated from this species. The results obtained from *in vitro* regeneration protocol showed that leaf and petiole explants were the most responsive explants and gave high percentage of response in forming micro shoots. Both explants were selected to obtain the optimum callus induction in Begonia. Previous experiments showed that callus induction could be obtained from leaf explants (Nakano *et al*., 1997; Jain, 1993), and young flower buds (Pierik and Tetteroo, 1987). Whereas Pierik and Tetteroo (1987) have found that young flower buds were capable of producing callus which were contrasting to callus from leaves of adult plants. Thus, comparing two different explants for callus induction in Begonia, suggested that leaf explants were more responsive and favourable than petiole explants.

In order to induce callus formation in Begonia, various factors need to be taken into consideration. The development of callus eventually depends on the origin and source of callus, duration of subculture, different types and concentrations of ingredients in the nutrient media and the effect of environmental factors such as photoperiod and temperature. However, amongst all factors involved, callus induction is normally depends

on the application of plant growth regulatosr. Auxin is known as the most promising plant growth regulator which always being used for callus induction and amongst all, 2,4-D has been recognized as the selected plant growth regulator. Omission of auxin led either to a rapid cessation of growth and cell division, or in suitable cultures, to organogenesis or embryogenesis. For example, Moghaddam and Taha (2005) found that in callus cultures of *Beta vulgaris* (sugar beet). Apart from that, Moghaddam *et al.*, (2000) also revealed that the use of TIBA as an anti-auxin increased organogenesis in sugar beet.

An efficient callus induction medium for Begonia was identified in the present study. The optimum yellowish callus (40.00-50.00%) was obtained in MS media supplemented with 0.1-0.6 mg/l 2,4-D (Table 3.1). The explants enlarged and callus tissues were initiated from the cut end of the explants. According to the present results, increasing 2,4-D concentration was also associated with a progressive reduction in the percentage of callus induction. Thus, in order to obtain optimum callus formation in Begonia, different combinations of BAP (0.1, 0.5, 1.0, 1.5 and 2.0 mg/l) and 2,4-D (0.1, 0.5, 1.0, 1.5 and 2.0 mg/l) were used in this study. The results also showed that the combinations of BAP and 2,4-D strongly enhanced green and yellowish callus induction and the optimum green callus was obtained in the MS media supplemented with 1.0 mg/l BAP and 0.1-0.5 mg/l 2,4-D (Table 3.2).

The results from Chapter 3 showed that the colour of the callus formed was more dependent on different concentrations of BAP and 2, 4-D than on the different types of explants used. Most of the callus was green, yellowish, compact and nodular in structure.

Thus, according to the present research in Begonia, the response of callus was more dependent on different concentrations of plant growth regulators applied in the media. Pigmentation is considerably influenced by the level of dextrose, presence of soluble starch, nitrogen deficiency, temperature, light and exogenous auxin (Narayanaswamy, 1977). Jain (1993) also managed to obtain yellow-green callus from *Begonia* x *elatior*. Green-pigmented callus grew better under light than in the dark (Narayanaswamy, 1977).

A lot of studies have been carried out on the formation of callus in different species of Begonia (Geng-guang *et al.,* 1983; Cassells and Morrish, 1985; Pierik and Tetteroo, 1987 and Nakano *et al*., 1997) . Geng-guang *et al.* (1983) managed to induce callus on SH and MS media supplemented with 2,4-D (0.1 mg/l), NAA (2.5 mg/l) and kinetin (KT) (0.25 mg/l) in *Begonia fimbristipula* Hence. Cassells and Morrish (1985) had also suggested that callus induction was optimum in the MS media supplemented with 0.001 mg/l 2,4-D and 0.75 mg/l 2,4-D in *Begonia rex*. Pierik and Tetteroo (1987) also managed to obtain callus in MS medium supplemented with BA (0.5 mg/l) and NAA (0.5 mg/l) in *Begonia venosa* Skan. Nakano *et al*. (1997) also found that NAA in rooting media stimulated both callus and root production in *Begonia* x *tuberhybrida* Voss.

Even though the regeneration capacity of the callus could be maintained for an unlimited period by subculturing the callus into fresh medium after four weeks incubation, Cassells and Morrish (1987b) had shown that the morphogenetic potential of callus decreased during subcultures, whereby callus derived from the sixth subculture could only produce only one-tenth the numbers of adventitious shoots from the initial callus. Based on the

current investigation, addition of 2,4-D in the media would be only suitable to induce yellowish and non-embryogenic callus. The explants managed to produce embryogenic green and non-embryogenic yellowish callus *in vitro* on MS media supplemented with combinations of BAP and 2, 4-D. The production of green embryogenic callus will be beneficial for somatic embryo development and suspension cultures in *Begonia* x *hiemalis* Fotsch. for subsequent mass propagation of this species.

Direct somatic embryogenesis was obtained by using leaf and petiole explants cultured on MS medium supplemented with 1.0mg/l BAP, 0.1-0.5mg/l 2,4-D, 3.0% sucrose and solidified with 0.2% gelrite. Although leaf and petiole from intact plants could induce micropropagation but leaf and petiole explants taken from *in vitro* plantlets were found to be better starting materials for Begonia micropropagation. By using *in vitro* plantlets as a donor plant, sterilization process and contamination problem can be solved easily. Previous research also recognized the application of in *vitro* grown seedlings (Lin *et al*., 1998; Moghaddam and Taha, 2005), tubercles (Castillo and Smith, 1997) and aseptic seedlings (Awal and Taha, 2008) as suitable starting materials in tissue culture procedures.

Although the formation of callus from plant tissue culture can be obtained easily, somehow the factors regulating callus proliferation are not thoroughly understood. Thus, in order to justify the effect of various factors involved, either from environmental, chemical or internal factors, some selections of the factors should be made. From this research, few chemicals, internal and environmental effects on direct somatic embryos induction were

examined. The factors involved the justification of the optimum media, the effect of explants and also the photoperiod effect on the cultures prior to the development of a new protocol for somatic embryos induction in Begonia.

The first attempt was focused on the effect of different ages (2-6-month-old) of *in vitro* plantlets on somatic embryo induction. The results showed that, increasing the age of plantlets caused the decrease in organogenesis for leaf and petiole explants (Figure 4.1). The results obtained also showed that 2-month-old of explants (both leaf and petiole) had significantly higher mean score (75.00%) than the other ages. Thus, 2-month-old plantlets were chosen for the purpose of callus and somatic embryos induction in this work. The results also showed that the second and the third position of leaf gave optimum responses on shoot production and rooting process as compared to other treatments.

Lin *et al.* (1998) observed that younger explants produced more shoots than older explants. They also observed that younger explants, which originally located closer to the stem apex, showed a higher percentage of shoot regeneration than the older explants, and this percentage significantly decreased with increasing positions. The results showed (Table 4.1, 4.2 and 4.3) that leaf explants gave high percentage of response in the overall media tested as compared with petiole segments, after 2 months being cultured. The results showed that 63.75% callus was obtained from leaf explants while 60.71% from petiole segments. The results were reported based on the percentage of callus obtained from each explant. Two types of callus were identified in this study i.e. embryogenic callus and non-embryogenic callus.

Various types of growth regulators such as 2,4-D, TDZ and BAP in combinations with 2,4-D were applied in this study. As reported in many other ornamental plants, somatic embryos induction was inhibited by the presence of 2,4-D. In Begonia, 2, 4-D alone, TDZ and combinations of cytokinins and 2,4-D were utilized. The results revealed that, 2,4-D and TDZ alone could not produce direct somatic embryogenesis in leaf and petiole explants. Successful somatic embryos induction was achieved with the presence of BAP and 2,4-D in the media. Based on Table 4.1 it was shown that MS media devoid of hormones did not give positive response on callus induction, whereas non-embryogenic callus was obtained in MS media supplemented with growth regulators (2,4-D and TDZ) (Table 4.2-4.3). However, different percentage of embryogenic and non-embryogenic callus were obtained in the MS media supplemented with BAP (0.1-1.0 mg/l) and 2,4-D (0.1-0.25 mg/l).

In the current investigation, the results showed that (Table 4.3) leaf and petiole explants managed to develop embryogenic and non-embryogenic callus after 2 months being cultured in the MS media supplemented with different concentrations of BAP, 2-iP, kinetin, zeatin, 2,4-D and other additives.

Previous research reported that 2,4-D and BAP affected the phase of callus induction through embryogenesis pathway. Nikam *et al.* (2003) identified that stem segments of *Agave sisalana* produced callus in MS media supplemented with BAP (1.0-1.5 mg/l) and 2,4-D (0.25-0.5 mg/l). Castillo and Smith (1997) also found that MS media supplemented

with 0.5 mg/l kinetin were capable of inducing direct somatic embryogenesis in *Begonia* x *gracilis* with no intervening callus stage. The results obtained from the current study showed that culturing leaf and petiole explants on MS medium supplemented with 1.0 mg/l BAP, 0.1-0.5 mg/l 2,4-D, 3% sucrose and solidified with 0.2% gelrite proved to be suitable for the production of somatic embryos and a new protocol could be developed from this research.

In the current investigation, direct somatic embryos induction was obtained after 2 months incubation on MS media supplemented with 1.0mg/l BAP, 0.1-0.5 mg/l 2,4-D, 3.0% sucrose and solidified with 0.2% gelrite. Greenish, nodular with globular stage of embryogenic callus (Plate 4.1) developed into torpedo and cotyledonary stage after 10-12 weeks of incubation period and finally developed into micro shoots. From observations, the heart shaped embryoid was not being able to be identified due to the development period was too fast and the development of embryoids was not synchronized.

The best induction medium for direct somatic embryogenesis of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red* was achieved on MS supplemented with 1.0 mg/l BAP, 0.1-0.5 mg/l 2,4-D, 3.0% sucrose and solidified with 0.2% gelrite. However, Castillo and Smith (1997) reported that 0.5 mg/l kinetin and 2.0% coconut water were effective in inducing direct somatic embryogenesis in *Begonia.* x *gracilis* explants.

In the current investigation, somatic embryogenesis and organogenesis in *Begonia* x *hiemalis* Fotsch. was obtained in MS supplemented with similar concentrations of BAP (1.0 mg/l) in combination with 2,4-D (0.1-0.5 mg/l) and NAA (1.0 mg/l). Somatic embryogenesis in Begonia employed 0.1-0.5 mg/l 2,4-D whereas 1.0 mg/l NAA was the optimum concentration for organogenesis. Thus, exogenous auxin and cytokinin were beneficial for somatic embryogenesis and organogenesis in Begonia.

Other than growth regulator treatments, different additives were also tested to obtain the best media for somatic embryo induction in Begonia. The results showed that the best induction of direct somatic embryogenesis of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red* was achieved on MS medium supplemented with 1.0 mg/l BAP, 0.5 mg/l 2,4-D, 500 mg/l casien hydrolysate, 3.0% sucrose, solidified with 0.2% gelrite (Awal and Taha, 2008).

Addition of casein hydrolysate in the callus induction medium was found to be beneficial and different concentrations of casein hydrolysate were also identified to optimize somatic embryo induction. The results showed that 100 mg/l and 500 mg/l casein hydrolysate produced high percentage of green nodular callus compared with other treatments after 2 months incubated under 16 hours light and 8 hours dark at 25 ± 1ºC. MS media supplemented with 1.0 mg/l BAP and 0.5 mg/l 2,4-D together with 100 mg/l casien hydrolysate produced 40.00% greenish globular callus after 2 months incubation whereas 500 mg/l casien hydrolysate produced 25.00% green callus by using leaf explants. Several reports also have proven that the use of casein hydrolysate as beneficial for the formation

of somatic embryos *in vitro* in *Gnetum edule* (Augustine and D'Souza, 1997) and interspecific hybrid of *Oryza* (Ling *et al*., 1983).

Prior to deriving a protocol for direct somatic embryo induction in Begonia, preliminary studies were carried out to identify the optimum concentrations of TIBA applicable in this study. The results showed that 1.0 mg/l TIBA was the optimum concentration for callus induction in Begonia (Table 4.7). Chen and Chang (2004) had also identified that direct application of TIBA (0.1 and 0.5 μM) significantly retard direct embryo formation in *Oncidium* 'Gower Ramsey'. Thus, this is in agreement with the present results which showed that the application of TIBA directly to the media would retard somatic embryogenesis process in the leaf explants, but at the same time induced the formation of embryogenic callus in Begonia provided TIBA was applied prior to culture of the leaf explants. Moghaddam and Taha (2005) also found that embryogenic callus of *Beta vulgaris* could be obtained by using *in vitro* seedlings treated with 1.0 μM TIBA.

The effects of different light treatment on the production of somatic embryogenesis were also investigated. The results showed that mean percentage of callus was not significantly different from different explants for different light treatment (Table 4.8). Dark incubation produced complete embryogenesis cycle compared with 16 hours light 8 hours dark incubation (Plate 4.2). Augustine and D'Souza (1997) also reported that callus incubation in the dark could give rise to a large number of immature embryos.

In the present study, a new protocol was developed to increase the quantity of embryogenic callus of Begonia (Table 4.9). The protocol proved that the formation and optimization of somatic embryos cultures were successfully obtained by using *in vitro* explants treated with 1.0 mg/l TIBA. The axenic plants derived from regeneration process were subcultured into MS media supplemented with 1.0 mg/l TIBA. The axenic plants were stunted and did not grow well as compared with control. Roots of these axenic plants showed a negative geotropism, grew upward and developed antagonistically from the normal root growth. The results indicated that anti-auxin property of TIBA disturbing the internal hormonal balance in the axenic plants. TIBA was known as auxin inhibitor and caused reduction in endogenous auxin activity in treated explants.

The results also revealed that the optimum medium produced yellowish embryogenic callus after being incubated in the dark for 8 weeks. The formation of embryogenic callus was further enhanced to develop embryoids for regeneration purposes. The embryogenic callus was further subcultured onto development medium for 8 weeks. Embryo development was achieved with GA_3 (0.5 mg/l). The results showed that the embryogenic callus developed into heart-shaped, torpedo-shaped and cotyledonary-stage embryos after 5-6 weeks incubated in the dark (Fig. 4.3-4.7). This results were also supported by the work of Gupta and Timmis (2005) whereby they managed to produce twenty to fifty cotyledonary embryos from 1.0 ml settled embryonal suspensor mass on development medium after 5-6 weeks incubation in the dark. During 4-5 weeks of incubation, frequencies of explants forming embryogenic callus were scored.

Microscopic identification of different stages of somatic embryogenesis were obtained using scanning electron microscope and the results were shown in Plates 4.3-4.7. Based on the results obtained, under scanning electron microscope, different stages of somatic embryos were observed developing from a mass of globular structures on the surface of leaf section after 10 weeks of incubation. During the process of somatic embryogenesis, the different stages of somatic embryos were developed starting from globular (Plate 4.4), heart-shaped (Plate 4.5), torpedo-shaped (Plate 4.6) and lastly followed by cotyledonary-stage (Plate 4.7). The length of each stage range from 400-700 μm (refer to Plate 4.3-4.7). The emerging globular started to split and form a curve at the end of one side and developed into heart-shaped stage.

From the current investigation, the globular stage and cotyledonary-stage were well distributed on the explant surfaces. The cotyledonary-stage was then successfully regenerated after being transferred to MS medium supplemented with 0.5 mg/l GA_3 and 0.2% activated charcoal. For development of somatic embryos into maturation, withdrawal of BAP and 2, 4-D from induction medium was necessary. The withdrawal of BAP and 2,4-D resulted in the growth of embryos into plantlets. In conclusion, the present work succeeded in inducing somatic embryos from leaf and petiole explants of Begonia and subsequently regeneration of embryogenic callus into complete plants.

The formation of embryogenic callus was further enhanced to initiate embryogenic suspension cultures. An adequate supply of nutrients is normally taken into consideration

to satisfy the demands for suspension cultures that favour the rapid growing of the callus. Thus, a protocol was developed to increase the quantity of callus in Begonia through the suspension cultures. The protocol established is shown in the Table 5.1, explained the formation and optimization of suspension cultures by using *in vitro* explants treated with 1.0 mg/l TIBA. TIBA is known as an auxin polar transport inhibitor (Chen and Chang, 2004). TIBA has been effective in inducing somatic embryogenesis in R*hizomatous irises* (Laublin *et al.*, 1991). However Chen and Chang (2004) had proven that direct application of TIBA (0.1 and 0.5 μM) significantly retarded direct embryo formation in *Oncidium* 'Gower Ramsey'. Moghaddam and Taha (2005) have also shown that embryogenic callus of *Beta vulgaris* could be obtained by using *in vitro* seedlings treated with 1.0 μM TIBA. Thus, from the results indicated that the application of TIBA directly to the media would retard somatic embryogenesis process in the leaf explants, but at the same time induced the formation of embryogenic callus in Begonia provided, TIBA was applied prior to culture of the leaf explants.

A successful new protocol was developed to induce and proliferate the embryogenic callus in the liquid media. The optimum liquid medium supplemented with 1.0 mg/l BAP and 0.1 mg/l 2,4-D was selected in this study. Razdan (1993) had also observed that a medium containing 2,4-D, induced the tissue multiplication but inhibited the maturation of the embryos.

The development of embryo-like structures from suspension cultures were induced by subculturing into MS solid media supplemented with 500 mg/l L-Proline for 6 weeks. The

emblings developed into *in vitro* plantlets after being subcultured into MS regeneration media. L-Proline which is which reacts as a precursor to the development of the emblings, one of the amino acids and helps during regeneration process.

The regeneration of emblings was achieved by subculturing the cotyledonary-like structure of the embryos into MS media supplemented with 0.2% (w/v) of activated charcoal. Activated charcoal is also reported to improve embryogenesis in carrot (Razdan, 1993), adsorbs a number of compounds including auxins and metabolites which often inhibited specific developmental stages of somatic embryos (Agarwal *et al.*, 2004). Even though at higher concentrations of activated charcoal (0.1, 0.2 and 0.4%) (w/v) was inhibitory and did not show embryo maturation in *Morus alba* L. (Agarwal *et al.*, 2004), but successful embryos development in Begonia were obtained in MS media supplemented with 0.2% (w/v) activated charcoal.

The somatic embryos produced was directly encapsulated using sodium alginate matrix to obtain synthetic seeds. The production of synthetic seeds in *Begonia* x *hiemalis* Fotsch. could overcome seedless or sterile plant problem. Elatior Begonia, a well known temperate hybrid plant which propagated through cutting and do not produce seeds. In the present work, synthetic seeds of *Begonia* x *hiemalis* Fotsch. were successfully produced.

Various factors were examined in line with the effort of producing artificial seeds. The factors including selection of propagules, selection of methods for ideal bead formation,

determination of the viability of the beads and also the effect of different storage period of the artificial seeds at 4 °C and lastly germination capability of the seeds.

In previous studies, different types of propagules were selected from the cultures including micro shoots, shoot buds, lateral buds, adventitious buds and somatic embryos. Synthetic seeds can be made using somatic embryos, micro shoots, protocorm-like bodies, shoot buds as the encapsulated propagules and most of the studies have been carried out using somatic embryos. Amongst the propagules, encapsulated somatic embryos were the most favored. In the current investigation, two different types of propagules were selected for synthetic seeds productions, i.e. cotyledonary-stage (somatic embryogenesis) and micro shoots (organogenesis).

To date, few studies have used micro shoots or multiple shoots for the production of artificial seeds as reported in pineapple (Soneji *et al.*, 2002). In Begonia, although somatic embryogenesis can be directly induced (Castillo and Smith, 1997) however organogenesis process have been largely favoured using explants such as leaf discs (Ringe and Nitsch, 1968; Roest *et al*., 1981; Cassells and Morrish, 1985), inflorescences (Pierik and Tetteroo, 1987), peduncles, petioles (Ringe and Nitsch, 1968; Cassells and Morrish, 1985) and tubers (Samyn *et al*., 1984). Thus, synthetic seeds production obtained from organogenesis process could also overcome regeneration problem in Begonia. The present research showed that synthetic seed was successfully developed from micro shoots *in vitro*. The optimum germination capability of the seeds prior to storage period was also determined.

The aim of this study was to obtain ideal synthetic seeds with isodiametric structure with high germination capability. Thus, the solid structure of the beads was also examined by using different concentrations of encapsulation agents. The germination rate of synthetic seeds was affected by various factors such as sodium alginate concentrations, different concentrations of $CaCl_2.2H_2O$ solution, different storage period of the beads in $CaCl_2.2H_2O$ solution, different types of sowing substrates and also storage period of the seeds. In the current investigation, micro shoots with 5.0-7.0 mm height were selected as propagules. The highest germination rate of micro shoots could be obtained with 3.0 % sodium alginate and maintained in 1.0 M $CaCl_2.2H_2O$ for 30 minutes.

Previous research showed that different types of encapsulation matrix could be used as coating agents but sodium alginate was found to be the most popular matrix for encapsulation matrix. Alginate was chosen as the encapsulation matrix because of its moderate viscosity, low toxicity, quick gelation and low cost (Onishi *et al.*, 1992). The germination and regeneration frequency of synthetic seeds was significantly affected by the concentration of sodium alginate, the concentration and duration of exposure to calcium chloride, $CaCl_2.2H_2O$ solution. Ipekci and Gozukirmizi (2003) identified that at low concentrations of sodium alginate (1.0-2.5%), uniform, sufficiently firm beads were not formed, resulting in a reduced frequency of germination. Castillo *et al.* (1998) identified that sodium alginate between 0.5-5.0% produced a sufficiently hard capsule while still maintaining embryo integrity of *Carica papaya* and a 20-30 min exposure to a complexing agent was required to achieve complete gelation. Ghosh and Sen (1994) found

that at a higher percentage of sodium alginate (6.0-7.0%), beads were harder which may have suppressed the ability of shoots and roots to emerge.

Hardening process of the synthetic seeds played an important role in the germination rate of the beads. An ideal bead formation was successfully achieved using complexation solution i.e. 1.0 M $CaCl_2.2H_2O$ solution for 30 minutes. Lisek and Orlikowska (2004) had identified the optimum bead production of strawberry and raspberry using 3.0% of sodium alginate and 0.75 M $CaCl_2.2H_2O$. Ipekci and Gozukirmizi (2003) also identified that somatic embryos of *Paulownia elongata* encapsulated with 3.0% of sodium alginate prepared in MS salt solution and submerged for 30 min in 0.50M $CaCl_2.2H_2O$ for hardening produced seeds with seed coats firm enough for handling and also allowed the seeds to convert to plantlets. Their finding is similar to the present work whereby for the complexation solution, 1.0 M $CaCl_2.2H_2O$ was selected in this study. However, Ghosh and Sen (1994) suggested that complexing time was longer (60-65 min) when using 0.25 M calcium as compared with a complexing time of 35-40 min for 0.5M. Ghosh and Sen (1994) also suggested that if 0.75M and 0.1M calcium were used, polymerization took place in 15-20 min.

Germination and survival rates of the seeds were determined using two different factors including different types of encapsulation matrix and different types of sowing substrates. The four encapsulating matrix tested during the preparative procedures (Table 6.2) indicated that the presence of growth regulators combinations affected the emergence of

the micro shoots. From the present investigation, it was found that the encapsulated micro shoots were successfully sown in MS basal medium and also in sterile garden soil.

Storage of micro shoots and somatic embryos in alginate beads resulted in 83.33-100% germination after 1-3 months of storage at 4 °C. The encapsulated micro shoots managed to germinate 53.00-83.00% and encapsulated somatic embryos germinated 6.67-50.00% with storage period of 90-120 days although it decreased. Castillo *et al.* (1998) examined the encapsulation procedure permitted somatic embryos of *Carica papaya* to survive storage for 85 days at 10°C under low light (8 $molm^{-2}s^{-1}$), whereas non-encapsulated (control) somatic embryos failed to germinate after storage. Ipekci and Gozukirmizi (2003) observed that low temperature (4 °C) storage gave promising results for survival and germination of somatic embryos of *Paulownia elongata*. Lisek and Orlikowska (2004) had also found that 90.00-100.00% survival of synthetic seeds of strawberry and raspberry after 3 months of storage at 4 °C. However, Janeiro *et al.* (1997) found that after one month storage at temperature at 20-22°C, the viability of encapsulated embryos of *Camellia japonica* cultured on germination medium had fallen from 90.00% to 13.00% and among the surviving embryos, only 6.0% and 18.00% underwent complete and incomplete germination, respectively. The results showed that synthetic seeds stored at 4 °C for 3 months retained their abilities to germinate and grow into normal plants.

Thus, the synthetic seeds obtained from encapsulation of micro shoots and somatic embryos can be used as a potential method to solve propagation problems in *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red* that have no seeds. The present work suggested

that the production of uniform beads with high frequency of germination would be useful for cloning and mass propagation especially for commercial purposes. Plantlets germinated by this procedure were morphologically identical to the donor material and developed normally under greenhouse conditions (Ipekci and Gozukirmizi, 2003).

Flowering is important for ornamental plants. Therefore in the present study, not only *in vitro* regeneration, callus induction, suspension cultures, somatic embryo induction and synthetic seeds production in Begonia had been investigated in this study but achievement pertaining to *in vitro* flowering of this species had also been examined. In order to induce *in vitro* flowering by using plant tissue culture technique, the environmental and internal factors need to apply *in vitro*. Tisserat and Galletta (1995) reported that flowering is a complex and systematic process involving combinations of environmental and genetic factors. Previous research showed that *in vitro* flowering has been reported in a number of plant species including *Pisum sativum* L. (Franklin *et al.*, 2000), *Bambusa edulis* (Lin *et al.*, 2003) and ginseng (Lin *et al.*, 2005). However, very limited number of reports concerning *in vitro* flowering are available in Begonia. In order to induce *in vitro* flowering in Begonia, various factors need to be taken into consideration and the induction of *in vitro* flowering eventually depends on the source of explants, different types and concentrations of plant growth regulators in the media, sucrose contents, adenine concentrations and the effect of environmental factors such as photoperiod and temperature. This study only focused on the effect of different types of explants, plant growth regulators combinations, chemicals and different dark incubation periods for optimizing the induction of *in vitro* flowering.

Very often, the use of plant growth regulators is necessary to induce growth and/or *in vitro* flowering (Tisserat and Galletta, 1988). In *Begonia franconis* LIEBM, a cytokinin proved to be necessary for the initiation and development of the floral organs (Berghoef and Bruinsma, 1979). However, Berghoef and Bruinsma (1979) found that cytokinin does not necessarily have a direct effect on the sex differentiation but they function primarily as a promoter of cell divisions. Nakano *et al.* (1999) also found that regenerated plants grew into the flowering stage did not show apparent morphological alterations. Pierik and Tetteroo (1987) also observed that during flowering, no deviations could be identified from the original genotype of *Begonia venosa* Skan.

Previous studies also showed that different types of media were selected to induce *in vitro* flowering. For example, Chambers *et al.* (1991) reported that *Dendrocalamus hamiltonii* produced flowers when cultured on MS medium supplemented with cytokinin. Chang and Chang (2003) also showed that *in vitro* flowering induction of terrestrial orchid *Cymbidium ensifolium* var. Misericors can be achieved on ½ MS medium supplemented with hormones. Saritha and Naidu (2007) also induced *in vitro* flowering using MS media supplemented with kinetin (0.5-4.0 mg/l) and IAA (0.1 mg/l). Thus, amongst all factors involved, *in vitro* flowering induction is normally depends on the application of plant growth regulators. MS medium supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA, 3.0% sucrose and 40 mg/l adenine were selected as an *in vitro* flowering induction media based on the most responsive media for *in vitro* regeneration in Begonia (Chapter 2). The induction of *in vitro* flowering by cytokinin has been reported for several plant species (Chambers *et al*., 1991; Lin *et al*. (2003); Taylor *et al*. (2005); Sim *et al*. (2007). Chambers

et al. (1991) found that flowering only occurred in treatments containing BA in the tested range, and not in growth regulator-free controls. Sim *et al*. (2007) had identified that, in the presence of coconut water, BA enhanced inflorescence stalk initiation and flower bud formation in *Dendrobium* Madame Thong-In. However, normal flower development was deformed in liquid medium but developed fully upon transferrring to two-layered medium (liquid over Gelrite-solidified). The presence of a cytokinins i.e. benzyladenine (BA), isopentenyladenine (2-iP) and zeatin in the media also is required for the induction of flowering in *Kniphofia leucocephala in vitro* as no inflorescences were observed in the control explants which received no cytokinin (Taylor *et al*., 2005).

Other than BAP, Lin *et al*. (2003) had also examined the effect of two different cytokinin i.e. TDZ and zeatin on *in vitro* flowering in *Bambusa edulis*. However, zeatin induced significantly less reproductive shoots than TDZ (0.5 μM). Whilst, Franklin *et al.* (2000) found that the presence of NAA or IBA (0.5 mg/l) combined with GA_3 (1.0 mg/l) in the medium enhanced the number of flowers. In conclusion, cytokinin such as BAP and zeatin is a common requirement for *in vitro* flowering induction.

Apart from that, three different explants including inflorescences, flower peduncles and petals were selected to induce *in vitro* flowering in Begonia and the explants were excised from actively growing plants (6-month-old). The results showed that direct *in vitro* flowering or reproductive shoots were obtained only from inflorescence explants and macro-morphological structures of the flowers were abnormal whereby no reproductive organs were developed in each floral organ except for the collectively organ known as the

calyx. Taha and Haron (2008) also found that *in vitro* flowering could only occur when cotyledons and shoots were used, but not from plantlets derived from stem and leaf explants of *Murraya paniculata*. (Jack) Linn. Daud (2006) also investigated that young influorescence of *Saintpaulia ionantha* Wendl. managed to induce 76.00% of *in vitro* flowering as compared with petal and petiole explants.

Addition of both adenine and sucrose resulted in floral initiation and floral development after 4 weeks. The results showed that 40 mg/l adenine would increase the number of *in vitro* flowering buds. Thus 40 mg/l adenine was selected for *in vitro* flowering induction in Begonia. The addition of adenine resulted in *in vitro* flowering and increased reddish colour shoot proliferation in Begonia. Ringe and Nitch (1968) had also showed that addition of adenine onto the media would initiate *in vitro* flowering in some varieties of Begonia. Daud (2006) also found that 40 mg/l adenine could also increase the percentage of flower buds of *Saintpaulia ionantha* Wendl. Takimoto (1960) had also identified that the plants receiving sugar may require only long dark periods for flower initiation. This is due to the supplemented sugar could replace the high-intensity light process.

In the present investigation, the effect of different sucrose concentration on *in vitro* flowering induction was also carried out by keeping the other parameters constant (plant growth regulators in the media, sucrose contents, adenine concentrations and the effect of environmental factors such as photoperiod and temperature). Among the different concentrations of sucrose tested in this study (1.0-5.0%), 5.0% sucrose induced the maximum numbers of abnormal flowers (13.75%). However, Franklin *et al.* (2000) had

reported that among the different concentrations of sucrose tested (0.0-6.0%), 3.0% sucrose induced the maximum numbers of flowers in green pea (*Pisum sativum* L.). Takimoto (1960) found that plants cultured on the medium containing 5.0% sucrose produced little chlophyll either in the light or in darkness. The increased sucrose supply to the meristem precedes the activation of energy-consuming processes such as mitotic activation and does not result from a higher demand by the meristem (Bernier *et al.*, 1993).

Based on the results obtained also showed that by culturing the explants for a short dark period i.e. 12 days (18.50%) were beneficial to *in vitro* flowering induction. Incubation of explants in the dark resulted in the production of a very low percentage of reproductive buds (18.50%) but a high percentage of vegetative buds (73.00%). Al-Khayri *et al.* (1991) had investigated the influence of photoperiod on *in vitro* flowering in spinach. Carson and Leung (1994) also had identified the effect of different range of photoperiods (0 hour light, 6 hours light, 12 hours light, 18 hours light and 24 hours light) including darkness on *in vitro* flowering by using the shoot cuttings as explants, which were cultured onto MS media devoid of exogenous plant growth regulators. They found that photoperiod did not appear to be a key-controlling factor for flower bud initiation in the shoot cuttings, as flower bud formation continued under all photoperiods. Even though continuous light (24 hours light) was most effective but it was not significantly so after 23 days of cultures of *Leptinella nana* L.

The ultimate aim of plant tissue culture technique is to transfer the regenerants back to natural environments. The survival rate of regenerants derived from different types of *in*

vitro techniques after being transferred to field or garden is of vital importance. The process of transferring to field or soil is termed as acclimatization. The successful acclimatization process will normally depends on morphological and environmental factors of the plantlets. The maintenance of both factors is crucial to increase the hardening percentage of micropropagated plants. In the present study, most of the plantlets regenerated from different types of *in vitro* treatments grew well in the greenhouse. The plantlets were grown in the plastic pot consisting of four different types of soil such as topsoil1, topsoil 2, sphagnum and vermiculite. The pots were kept covered with a plastic to avoid excessive water loss and to maintain high humidity. Then, the pots were kept in the culture room for 3-4 weeks before transferring the plantlets to the greenhouse and the plants were watered once every day. The hardening process was successful whereby the plant height increased rapidly during 6-9 months of transferring them to the garden soil.

In the current work, the plants were successfully acclimatized (80.00%) and developed into healthy plants after being transferred onto topsoil 1, topsoil 2, sphagnum and vermiculite in the greenhouse (Table 7.1). The results also showed that the topsoil 1 was the optimum substrate for acclimatization. Topsoil 1 is the normal conventional soil, which was bought from local plant nursery in Sungai Buloh, Selangor. Thus, topsoil 1 was selected as a substrate for other experiments. *In vitro* plantlets derived from different types of treatments including from *in vitro* regeneration, suspension cultures, somatic embryogenesis, synthetic seeds and *in vitro* flowering were transferred onto topsoil 1. The survival rate or most of the regenerants derived from different types of *in vitro* process acclimatized into topsoil 1 in the greenhouse were more than 80.00% except for the regenerants which were germinated from the synthetic seeds production (36.67%-56.67%) (Table 7.2).

During hardening process, preacclimatization of the plantlets was extremely important prior to transferring them into soil. Preece and Sutter (1991) found that plantlets undergo a rapid and extreme change in physiological functioning when they are removed from *in vitro* culture to soil mixture. With plants grown in test tubes the cuticle (wax layer) is often poorly developed, because the relative humidity is often 90-100% *in vitro* (Pierik, 1987). Gangopadhyay *et al.* (2002) also found that the *in vitro* propagated plants posses thin, soft, photosynhetically hypoactives leaves having less epicuticular wax, trichomes and malfunctioned stomata resulting in excessive transpiration. During acclimatization to *ex vitro* conditions, leaf thickness generally increases, leaf mesophyll progresses in differentiation into palisade and spongy parenchyma, stomatal density decreases and stomatal form changes from circular to elliptical one (Pospisilova *et al.*, 1999).

It is well known that root induction during micropropagation process is stimulated by endogenous or exogenous auxins. The current investigation showed that most of the plantlets managed to induce roots directly from each treatment including regeneration process, somatic embryogenesis, synthetic seeds production and *in vitro* flowering. The survival rate of the plantlets after being transferred to the acclimatization media were also identified.

CHAPTER 9

9.0 CONCLUSION

Micropropagation studies of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red* was successfully carried out in the present work which involved the process of *in vitro* regeneration, callus induction, initiation of suspension cultures, the process of indirect and direct somatic embryos induction, formation, storage period and germination capability of the synthetic seeds, *in vitro* and *ex vitro* flowering induction and finally acclimatization or hardening process. From this study, four different intact explants of selected Begonia gave similar response on *in vitro* micro shoots formation after being cultured for 8 weeks onto MS media containing various concentrations of BAP (0.1-5.0 mg/l) and NAA (0.1-3.0 mg/l). From this study, 1.0 mg/l BAP in combination with 1.0 mg/l NAA and 1.0 mg/l Zeatin and 1.0 mg/l NAA were found to be the best combination of growth regulators to be supplemented to MS medium in obtaining optimum regeneration in *Begonia* x *hiemalis* Fotsch cv. *Schwabenland Red.* The results obtained from *in vitro* regeneration protocol showed that leaf and petiole explants were the most responsive explants and gave high percentage of response (68.67% and 51.00%) in forming normal micro shoots.

Apart from achievement of *in vitro* regeneration in Begonia, callus induction was also investigated on this species. An efficient callus induction medium for Begonia was identified in the present study. The optimum yellowish callus (40.00-50.00%) was obtained in MS media supplemented with 0.1-0.6 mg/l 2,4-D. The results also showed that the combinations of BAP and 2,4-D strongly enhanced green and yellowish callus

formation and the optimum green callus was obtained in the MS medium supplemented with 1.0 mg/l BAP and 0.1-0.5 mg/l 2,4-D.

Direct somatic embryogenesis was achieved by using leaf and petiole explants cultured on MS medium supplemented with 1.0mg/l BAP, 0.1-0.5mg/l 2,4-D, 3.0% (w/v) sucrose and solidified with 0.2%(w/v) gellum gum (Gelrite). Although leaf and petiole from intact plants could induce micropropagation but leaf and petiole explants taken from *in vitro* plantlets were found to be better starting materials for micropropagation of Begonia. Different percentage of embryogenic and non-embryogenic callus were obtained in the MS media supplemented with BAP (0.1-1.0 mg/l) and 2,4-D (0.1-0.25 mg/l). In the current investigation, the results showed that (Table 4.3) leaf and petiole explants managed to develop into embryogenic and non-embryogenic callus after 2 months being cultured in the MS media supplemented with different concentrations of BAP, 2-iP, kinetin, zeatin, 2,4-D and other chemicals. The best induction medium for direct somatic embryogenesis of *Begonia* x *hiemalis* Fotsch.cv. *Schwabenland Red* was achieved on MS supplemented with 1.0 mg/l BAP, 0.1-0.5 mg/l 2,4-D, 500 mg/l casein hydrolysate, 3.0% (w/v) sucrose and solidified with 0.2% (w/v) gellum gum (Gelrite).

Embryo development was achieved with combination of BAP, NAA (1.0 mg/l) and GA_3 (0.5 mg/l). The results showed that the embryogenic callus developed into heart-shaped, torpedo-shaped and cotyledonary-stage of embryos after 5-6 weeks incubated in the dark. Microscopic identification of different stages of somatic embryogenesis were obtained using scanning electron microscopy and the cotyledonary-stage was then successfully

regenerated after being transferred to MS medium supplemented with 0.5mg/l GA_3 and 0.2% (w/v) activated charcoal. In conclusion, the present work succeeded in inducing somatic embryos from leaf and petiole explants of Begonia and subsequently regeneration of embryogenic callus.

The synthetic seeds obtained from encapsulation of micro shoots and somatic embryos can be used as a potential method to solve propagation problems in *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red* that have no seed. The present work suggested that the production of uniform beads with high frequency of germination would be useful for cloning and mass propagation especially for commercial purposes.

MS medium supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA, 3.0% (w/v) sucrose and 40 mg/l adenine were selected as an *in vitro* flowering induction medium based on the most responsive media for *in vitro* regeneration in Begonia. The results showed that direct *in vitro* flowering or reproductive shoots were obtained only from inflorescence explants and macro-morphological structures of the flowers were abnormal whereby no reproductive parts were developed in each floral organ except for the collectively organs known as the calyx.

In the current work, the plants were successfully acclimatized (80.00%) and developed into healthy plants after being transferred to topsoil 1, topsoil 2 and sphagnum in the green house. The results also showed that the topsoil 1 was the optimum substrate for

acclimatization. Topsoil 1 is the normal conventional soil which was bought from local plant nursery in Sungai Buloh, Selangor. Thus, topsoil 1 was selected as a substrate for other experiments. *In vitro* plantlets derived from different types of treatments were transferred onto topsoil 1. The survival rate of most of the regenerants derived from different types of *in vitro* process including from *in vitro* regeneration, somatic embryogenesis and synthetic seeds were acclimatized into topsoil 1 in the green house were more than 80.00% except for the regenerants which were germinated from the synthetic seeds production (36.67% - 56.67%).

Thus, future work such as cellular studies on *in vivo* and *in vitro* plants, effect of radiation on *in vitro* flowering and some molecular aspect such as protein content etc will be suitable for this species. Apart from that, protoplast and transformation work in Begonia should also be done to identify another aspect in Begonia.

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APPENDIX 1

JOURNALS/PROCEEDINGS:

1. Taha, R.M., Rashid, Z.S. and **Awal, A.** *In vitro* flowering of *Begonia* x *hiemalis* Fotsch. cv. *Schwabenland Red*. Annals of Applied Biology (in press).

2. **Awal, A.**, Taha, R.M. and Hasbullah, N.A. (2008). Induction of somatic embryogenesis and SEM in *Begonia* x *hiemalis* Fotsch. *in vitro*. Proceedings of the 6th. International Symposium on *In Vitro* Culture and Horticultural Breeding. Acta Horticulturae, No: 829.

3. **Awal, A.**, Taha, R.M. and Hasbullah, N.A. (2008). Induction of somatic embryogenesis and plant regeneration in *Begonia* x *hiemalis* Fotsch. *in vitro*. J. Bio. Sc., 8(5): 920-924.

4. **Awal, A.**, Taha, R.M. and Hasbullah, N.A. (2007). Artificial Seed Formation from Micro Shoots of *Begonia* x *hiemalis* Fotsch. *in vitro*. International Journal of Environmental Sciences-Catrina 2(2): 189-192.

5. Hasbullah, N.A., Taha, R.M. and **Awal, A.** (2008). Production of synthetic seeds from micro shoots and somatic embryos of *Gerbera jamesonii* Bolus ex. Hook f. *in vitro*. Proceedings of the 6th. International Symposium on *In Vitro* Culture and Horticultural Breeding. Acta Horticulturae, No: 829.

6. Hasbullah, N.A., Taha, R.M. and **Awal, A.** (2008). Growth optimization and organogenesis of *Gerbera jamesonii* Bolus ex. Hook f. *in vitro*. Pakistan J. of Bio. Sc., 11(11): 1449-1454.

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AWARDS:

1. **BRONZE MEDAL:**

   Taha, R.M., Daud, N., Subramaniam, G. and **Awal, A.** (2004). Production of Synthetic Seeds from Horticultural Plants. Expo Science, Technology and Innovation 2004. 27-29 August 2004.

2. **GOLD MEDAL:**

   Taha, R. M., Daud, N., **Awal, A.**, Hasbullah, N. A. and Mokhtar, A. (2006). Production of Synthetic Seeds from Somatic Embryos of Selected Ornamental Plants. 3rd International Biotechnology Trade Exhibition, Conference and Awards (Biotechnology Asia 2006). August 2006.

3. **SILVER MEDAL:**

   Taha, R. M., Daud, N., **Awal, A.**, Hasbullah, N. A. and Mokhtar, A. (2006). Production of Synthetic Seeds from Somatic Embryos of Selected Ornamental Plants. Seoul International Invention Fair 2006. 7-11 December 2006.

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1. Taha. R.M., Daud, N., Subramaniam, G. and **Awal, A.** (2004). Production of Synthetic Seeds from Horticultural Plants. Expo Science, Technology and Innovation 2004. 27-29 August 2004.

2. **Awal, A.** and Taha, R.M. (2005). Production of artificial seeds of *Begonia* x *hiemalis* Fotch *in vitro*. Graduate Event University of Malaya. 11-12 May 2005.

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9. **Awal, A**., Taha, R.M., Hasbullah, N.A. and Mokhtar, A. (2006). Plant Regeneration from Encapsulation of Microshoots of Selected Ornamental Plants. The 11th Biological Science Graduate Congress, Faculty of Science, Chulalongkorn University, Thailand. 14-17 December 2006.

10. Hasbullah, N.A, Taha, R.M, **Awal, A.** and Mokhtar, A. (2006). Production and Identification of Different Stages of Somatic Embryos from Selected Ornamental Plants. The 11th Biological Science Graduate Congress, Faculty of Science, Chulalongkorn University, Thailand. 14-17 December 2006.

11. **Awal, A.**, Taha, R.M. and Hasbullah, N.A. (2007). Artificial Seed Formation from Micro Shoots of *Begonia* x *hiemalis* Fotch. *in vitro*. The 2nd International Conference of The Egpytian Society for Environmental Sciences: The Role of Genetics and Biotechnology in Conservation of Natural Resources. Ismailia, Egypt. 9-10 July 2007.

12. Hasbullah, N.A., Taha, R.M. and **Awal, A.** (2007). Identification of Embryogenic Callus and Somatic Embryo Formation in *Gerbera jamesonii* Bolus ex. Hook f. *in vitro*. The 2nd International Conference of The Egpytian Society for Environmental Sciences: The Role of Genetics and Biotechnology in Conservation of Natural Resources. Ismailia, Egypt. 9-10 July 2007.

19. **Awal, A.**, Taha, R.M. and Hasbullah, N.A. (2008). Induction of somatic embryogenesis and SEM in *Begonia* x *hiemalis* Fotsch. *in vitro*. The 6th International Symposium on *In Vitro* Culture and Horticultural Breeding: 2020 Vision for *In Vitro* Horticultural breeding. Brisbane, Queensland, Australia, 24-28 August 2008.

20. Mat Taha, R., Hasbullah, N.A. and **Awal, A.** (2009). *In vitro* flowering of selected ornamental plants. 2th International Conference on Landscape and Urban Horticulture. 9-13 June 2009, Bologna, Italy.

21. Hasbullah, N.A., Mat Taha, R., and **Awal, A.** (2009). *In vitro* plantlets from somatic embryos of selected ornamental plants - a new prospect for interior decorations and horticulture industry. 2th International Conference on Landscape and Urban Horticulture. 9-13 June 2009, Bologna, Italy.

22. Rosna, M.T., Abdullah, S., Hasbullah, N.A. and **Awal, A.** (2009). Micropropagation of selected ornamental plants. 23rd EUCARPIA Symposium-Section Ornamentals "Colourful Breeding and Genetics". 31 August-4 Sept 2009, Leiden, The Netherlands.

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In vitro Formation of Synthetic Seed from Microshoots of *Begonia* x *hiemalis* Fotch.

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ABSTRACT

Artificial seed or synthetic seed formation of *Begonia* x *hiemalis* Fotch var. *Schwabenland Red* had been induced from leaf explants *in vitro*. The explants were cultured on (Murashige and Skoog (MS) medium supplemented with combinations of 1.0 mg/l Benzylamino purine (BAP) and 1.0 mg/l Naphthalene acetic acid (NAA). After 8 weeks, regenerated microshoots were excised from leaf cultures and microshoots successfully encapsulated in 3% sodium alginate solution together with MS salt solution and polymerized within 1.0M $CaCl_2.2H_2O$ solution for 30 minutes. The beads produced were with firm coats, round beads and uniform size and in good shape for handling. The results demonstrate that the optimum germination and survival rate of encapsulated matrix was in MS medium containing 3% sodium alginate solution supplemented with 3% sucrose, 1.0 mg/l BAP in combination with 1.0 mg/l NAA. The viability of the encapsulated micro shoots after storage period at 4 °C was also determined. High germination rate (100%) was achieved after 1-3 months storage whereas low germination rate (7-53%) was obtained after 4-6 months storage. The seeds were also successfully germinated in three different germination media including MS basal, garden soil and vermiculite.

Keywords: *Begonia* x *hiemalis* Fotch., encapsulated microshoots, synthetic seeds, tissue culture.

INTRODUCTION

Synthetic seed technology has benefited the mass production of plant propagation system *in vitro*. The production of synthetic seeds in tissue culture system could overcome breeding problems in seedless plants. In order to obtain the exact form of synthetic seed, specific applications might be necessary. Such encapsulation provides protection to the microshoots and allows an advisable handling similar to the natural seeds.

Begonias are normally grown as ornamental plants especially as decorative houseplants and for landscaping. Begonias are unique for their sheer beauty and variety of leaves. Other than great horticultural value, Begonias also has medicinal values. It is estimated that there are about 10,000 Begonias hybrids and cultivars worldwide. One of the most popular hybrids of Begonias is *Begonia* x *hiemalis* Fotch., a temperate plant that is commercially used as flower potting plant and propagated by cuttings due to the unavailability of the seeds.

Generally, synthetic seeds production can be induced using somatic embryos, microshoots, protocorm-like bodies, shoot buds as the encapsulated propagule and most of the studies have been carried out using somatic embryos. To date, few studies have used microshoots or multiple shoots for the production of artificial seeds as reported in pineapple (Soneji *et al*, 2002). In Begonia, although somatic embryogenesis can be directly induced (Castillo and Smith, 1997) but organogenesis process have been largely favoured using explants such as leaf discs (Ringe and Nitsch, 1968; Roest *et al*., 1981; Cassells and Morrish, 1985), inflorescences (Pierik and Tetteroo, 1987), peduncles, petioles (Ringe and Nitsch, 1968; Cassells and Morrish, 1985) and tubers (Samyn *et al*., 1984). Thus, synthetic seeds production obtained from organogenesis process could also overcome regeneration problem in Begonia.

The main objective of the present study was to develop a procedure for the encapsulation of in vitro-derived microshoots of *Begonia* x *hiemalis* Fotch., which do not form seeds, in alginate for the production of synthetic seeds, as a new propagation method. Five different factors were identified in this study including concentration of sodium alginate, concentration and duration of exposure to $CaCl_2.2H_2O$ solution, presence or absence of hormone in the encapsulation solvent, different types of sowing media and cold storage periods of the artificial seeds.

MATERIALS AND METHODS

Preparation of culture media and microshoots

The explants sources consisting of small pieces of leaves derived from *in vitro* plantlets were established on MS medium (Murashige and Skoog, 1962) supplemented with 30 g/l sucrose and 2.5 g/l gelrite added with 1.0 mg/l BAP in combination with 1.0 mg/l NAA. The pH of the medium employed in the experiment was adjusted to 5.8 sterilization process at 121°C for 21 minutes. Cultures were kept in culture room at 25 ± 1°C, under 16-h light photoperiod of light intensity (1000 lux). Microshoots (approx. 3 mm in length) were excised from cultures after 8 weeks in culture (Fig. 1B). The microshoots were carefully isolated and were blot dried on filter paper. After being encapsulated, these microshoots will be used as artificial seeds.

Formation of beads

The ideal procedure for encapsulation of synthetic seeds were identified by studying the effect of various factors on bead formation which include: (1) concentration of sodium alginate, (2) concentration and duration of exposure to $CaCl_2.2H_2O$ solution,

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(3) presence or absence of hormone in the encapsulation solvent, (4) different types of sowing media and (5) storage period of the artificial seeds. For the production of synthetic seed in *Begonia* x *hiemalis* Fotch., microshoots were mixed in the encapsulating matrix which consisted of 2-5% solutions of sodium alginate (Sigma), mixed up in MS basal medium solution (pH 5.8) added with 30mg/l sucrose and 1.0mg/l BAP in combination with 1.0mg/l NAA. Apart from that, the microshoots were also encapsulated with sodium alginate solution devoid of MS basal salts and plant growth regulator. Subsequently, by using sterile micropipette, the microshoots were drawn up with some encapsulation matrix and dropped into the matrix solution ($CaCl_2.2H_2O$ solution). Different concentrations of $CaCl_2.2H2O$ solution (0.1 - 1.0M) were also identified. The seeds were left to harden for a certain period (10-30 min) for complexation. Then, the seeds were washed in MS standard liquid medium avoiding from sticking together and retrieved using nylon mesh. The resulting capsules or beads consisted of one propagule/ bead.

Germination of beads and storage

The beads were germinated on various germination media and substrates, for germination evaluation. Germination media including MS basal media devoid of sucrose (control), MS basal media supplemented with 3% sucrose, garden soil, vermiculite and also sphagnum peat. All the germinating substrates were prepared in the jam jar and autoclaved prior to use. The cold-storage of the beads were also done in the incubator at 4°C from 1-6 months prior to germination process. All the samples were germinated and incubated under the culture room conditions at 25 ± 1°C, under 16-h light photoperiod of light intensity (1000lux). The germination days of the synthetic seeds were recorded manually and germination rate were recorded after 6 weeks of germination.

RESULTS

Effect of alginate matrix on beads formation

Microshoots were successfully encapsulated in 3% sodium alginate. The alginate solution prepared in MS salt solution together with 3% sucrose was left to harden for another 30 min in 1.0M $CaCl_2.2H_2O$, which produced beads with firm coats, round beads and uniform size and in good shape for handling (Fig. 1C). The effect of various concentrations of sodium alginate (2-6%) and calcium chloride (0.25-1.25M) for bead formation from encapsulated microshoots is presented in Table 1. Lower concentrations of sodium alginate (1-2%) formed fragile beads with no definite shapes. At higher concentrations (more than 4%) the beads were isodiametric in shape but too hard. Observations were made after 30 min in $CaCl_2.2H_2O$ solution for hardening process. However, it was found that encapsulated microshoots showed different degree of successes based on ideal beads produced. Further experiments were carried out in order to determine the optimal complexation of encapsulation matrix. In the present study, it was observed that MS medium containing 3% sodium alginate solution supplemented with 3% sucrose, 1.0 mg/l BAP in combination with 1.0 mg/l NAA maintained in 1.0M $CaCl_2.2H_2O$ solution for encapsulation was the optimal concentration to produce artificial seeds from encapsulated microshoots. The beads started to germinate after 7 days in culture with 90.48% germination rate and developed into plantlets 10 days later (Fig. 1D).

Regrowth of beads

The optimum seedling rate of synthetic seeds under sterile conditions was 90.48% (Table 2). To evaluate the germination of beads is to observe the increase in size of explants, with breakage of the capsule and extrusion of the shoot or of a leaf bud. The explants were considered alive if they remained green, with no necrosis or yellowing and continued to enlarge after encapsulation.

Table (1): Effect of different concentrations of sodium alginate and calcium chloride on beads formation. Observations were taken after 30 min of complexation process in $CaCl_2.2H_2O$ solution.

$CaCl_2.2H_2O$ (M)	Sodium alginate concentration (%)				
	2.0	3.0	4.0	5.0	6.0
0.25	+	+++	+++	+++	+++
0.50	+	+++	+++	+++	+++
0.75	++	+++	+++	+++	++++
1.00	++	++++	++++	++++	++++
1.25	++	++++	++++	++++	++++

\+ Very fragile bead with no definite shape
++ Fragile beads with no definite shape
+++ Soft, solid and uniform shape
++++ Optimal, firm, uniform and round shape

Table (2): Growth response of microshoots of Begonia encapsulated in different capsule matrix after being transplanted into MS media for 10 and 30 days. Unencapsulated microshoots were used as controls.

Capsule matrix	Germination rate (%) (after 10 days)	Survival rate (%) (after 30 days)
Control	80.00 ± 0.08	80.00 ± 0.08
Ca-free MS + dist. water	80.95 ± 0.06	30.00 ± 1.58
Ca-free MS + 3% sucrose	90.48 ± 1.08	64.00 ± 1.20
Ca-free MS + 3% sucrose + 1mg/l BAP + 1mg/l NAA	90.48 ± 1.08	83.33 ± 0.06

Effect of sowing media on germination

Table 3 demonstrated that most of the beads managed to survive after 8 weeks in culture on MS basal medium (100%) and sterile garden soil (83.33%). The survival rate of seedlings reached 80.0% after they were transferred to pots containing garden soil.

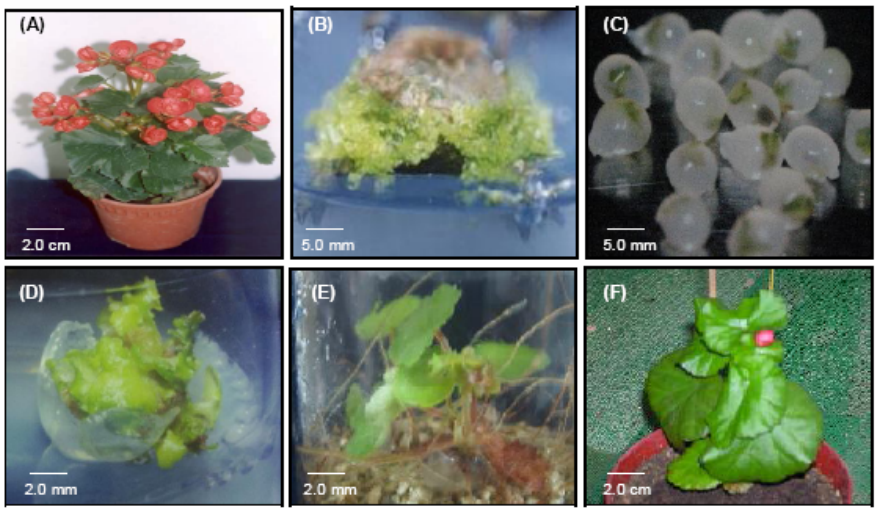

Figure (1): (A) the intact plant of *Begonia* x *hiemalis* Fotch. var. *Schwabenland Red.*, **(B)** microshoots derived from leaf explants after 8 weeks in culture, **(C)** microshoots encapsulated in alginate matrix, **(D)** after 7 days, plantlet emerged from artificial seed on germination medium, **(E)** synthetic seeds germinating on vermiculite after 3 months, and **(F)** Plantlet regenerated from artificial seeds produced flower after 6 months transferred into soil.

Storage period effects

Plantlet regeneration from cold-stored synthetic seeds was morphologically similar from regeneration cultures. The results in table 4 showed that 96.67-100% germination rate could be obtained from 0-60 days storage, whereas 90 days storage also showed precocious germination and the seeds germinated at 83.33%. The 120-150 days storage seeds showed 7-54% germination. Most of the artificial seeds developed into normal seedlings. No phenotypic changes were observed from the plantlets and over 90% of the plantlets developed into healthy, field-grown plants with about 5 cm height after 4 months cultured into MS basal medium. After 9 months of acclimatization process, a number of plants produced flower (Fig. 1F).

Table (3): Effect of different sowing media on germination rate of synthetic seeds of Begonia. Unencapsulated microshoots were used as controls.

Sowing medium	Germination rate (%)
Control	80.00 ± 0.08
MS + 3% sucrose	100.00 ± 0.00
Garden soil	83.33 ± 0.06
Vermiculite	56.67 ± 1.20
Sphagnum	36.67 ± 1.18

Table (4): Effect of storage time (days) at 4°C on germination of synthetic seeds on MS basal medium.

Day storage	Germination rate (%)
0	100 ± 0.00
30	96.67 ± 0.03
60	100 ± 0.00
90	83.33 ± 0.06
120	53.33 ± 1.00
150	6.67 ± 0.00
180	0

DISCUSSION

The present study showed that synthetic seed was successfully developed from microshoots *in vitro*. The optimum germination capability of the seeds prior to storage period was also identified. The germination rate of synthetic seed was affected by various factors such as sodium alginate concentrations, different concentrations of $CaCl_2.2H_2O$ solution, different types of sowing substrates and also storage period of the seeds. The highest germination rate of microshoots can be obtained with 3% sodium alginate and maintained in 1.0M $CaCl_2.2H_2O$ for 30 minutes. Previous research showed that different types of encapsulation matrix could be used as coating agents but sodium alginate was as the most popular for encapsulating matrix. Redenbaugh *et al.* (1986) also identified that the production of sodium alginate from different commercial sources may be due to differential purity of alginic acid or the variation in the mannuronic acid: guluronic acid ratio. Alginate was chosen as the encapsulation matrix because of its moderate viscosity, low toxicity, quick gelation and low cost (Onishi *et al*, 1992).

Hardening process of the synthetic seeds carried an important role in the germination rate of the beads. An ideal bead formation was successfully achieved using complexation solution ie. 1.0M $CaCl_2.2H_2O$ solution for 30 minutes. Lisek and Orlikowska (2004) had also identified the optimum bead production of strawberry and raspberry using 3% of sodium alginate and 0.75M

$CaCl_2.2H_2O$. Their finding is similar to the present work except for the complexation solution whereby 1.0M $CaCl_2.2H_2O$ was selected in this study.

Germination and survival rate of the seeds were identified using two different factors including different types of encapsulating matrix and different types of sowing substrates. The four encapsulating matrix tested during the preparative procedures (Table 2) indicated that the presence of hormonal combinations affected the emergence of the microshoots. From the present investigation, it was found that the encapsulated microshoots were successfully sown in MS basal media and sterile garden soil.

Storage of microshoots in alginate beads results in 96.67-100% survival after 60 days storage at 4 °C. The synthetic seeds managed to germinate 53-83% with storage period of 90-120 days although it decreases multiplication of Begonia. Lisek and Orlikowska (2004) had also observed 90-100% survival of synthetic seeds of strawberry and raspberry after 90 days (3 months) in storage at 4°C. The results showed that the synthetic seeds stored at 4°C for 3 months retained their ability to germinate and grow into normal plants.

In conclusion, the synthetic seeds production obtained from encapsulation of microshoots can be used as a potential method to solve problems of propagation for *Begonia* x *hiemalis* Fotch. var. *Schwabenland Red* that have no seed. The results obtained showed that encapsulated explants could be handled like true seeds. Furthermore, plantlets produced from synthetic seeds have been successfully transferred to soil for hardening and managed to survive as any other normally field grown plant. The present work suggested that the production of uniform beads with high frequency of germination would be useful for cloning and mass propagation especially for commercial purposes.

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Induction of Somatic Embryogenesis and Plant Regeneration in *Begonia* × *hiemalis* Fotsch. *in vitro*

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Abstract: Direct somatic embryogenesis induction of *Begonia* × *hiemalis* Fotsch. (Elatior Begonia) was initiated from two different explants i.e., leaves and petioles. Both explants were cultured on MS medium supplemented with different concentrations of Benzylaminopurine (BAP) and 2,4-Dichlorophenoxyacetic acid (2,4-D). The results showed that combinations of 0.5-1.0 mg L^{-1} BAP and 0.1 mg L^{-1} 2,4-D produced direct somatic embryogenesis from leaf and petiole explants. Different concentrations of casein hydrolysate were also tested to optimize somatic embryo induction. The results showed that 100 mg L^{-1} casein hydrolysate could produce 53.08% nodular callus and 24.16% green embryogenic callus, whereas 500 mg L^{-1} casein hydrolysate produced 30.83% nodular callus and 23.75% green embryogenic callus. The embryogenic callus were then transferred to MS medium supplemented with 0.5 mg L^{-1} Gibberelic Acid (GA_3) with 0.2 g L^{-1} activated charcoal for further embryogenesis development and further regeneration.

Key words: Direct somatic embryogenesis, casein hydrolysate, activated charcoal, *Begonia* × *hiemalis* Fotsch.

INTRODUCTION

Somatic embryogenesis is the process by which somatic cells differentiate into somatic embryos (Arnold *et al*., 2002) through characteristic embryological stages without fusion of gametes (Schumann *et al*., 1995). Somatic embryogenesis is one of the most important methods in plant propagation. Embryogenesis capacity is influenced by cultural conditions, genotype and their interaction (Petitprez *et al*., 2005). For the initiation of a tissue, capable of somatic embryogenesis, a basic requirement is the presence of an auxin (Gairi and Rashid, 2004). All plantlets obtained through somatic embryogenesis did not differ phenotypically from the parental clones (Stefaniak, 1994).

Begonias are normally grown as ornamental plants especially as decorative houseplants and for landscaping. Begonias are unique for their sheer beauty and variety of leaves. It is estimated that there are about 10 000 Begonias hybrids and cultivars worldwide. Other than great horticultural value, Begonias also has medicinal values. *Begonia* × *hiemalis* Fotsch. (Elatior Begonia) is a temperate plant, which is commercially used as flower potting plant and does not produce seeds.

Although *in vitro* regeneration systems have been established for Begonia, *Begonia* × *hiemalis* Fotsch. (Appelgren, 1985; Cassells and Morrish, 1985; Pierik and Tetteroo, 1987), somatic embryogenesis induction has not been defined. Direct somatic embryogenesis in Begonia was induced using leaf and petiole explants *in vitro*. The aim of this study was to identify the optimum media for direct somatic embryo induction of Begonia *in vitro* from two different types of explants. Apart from that, the effects of casein hydrolysate and dark treatment on somatic embryogenesis were also investigated. The development and germination of *in vitro* plantlets derived from somatic embryos were also discussed.

MATERIALS AND METHODS

Intact stock plants of *Begonia* × *hiemalis* Fotsch. var. *Schwabenland Red* obtained from local nursery were grown in the culture room under 16 h photoperiod at 25±1°C. Standard tissue culture methods were used. The healthy leaf explants were collected from the stock plants purchased from nursery, surface sterilized and cultured onto regeneration medium MS (Murashige and Skoog, 1962) medium supplemented with 1.0 mg L^{-1} BAP and 1.0 mg L^{-1} NAA) to produce *in vitro* plantlets. The plantlets were maintained at 25±1°C and further subcultured every 6 weeks.

Two different explants including young leaves and petioles were selected from the *in vitro* plantlets. The leaf explants were cut approximately 0.5×0.5 cm whereas the

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petiole explants were cut into 0.5 cm long and then they were cultured onto MS medium supplemented with different concentrations of BAP (0.1, 0.5, 1.0, 1.5 and 2.0 mg L^{-1}) and 2,4-D (0.1, 0.5, 1.0, 1.5 and 2.0 mg L^{-1}) for embryogenic callus induction.

The optimum medium for embryogenic callus induction was used to study the effect of different concentrations of casein hydrolysate on somatic embryo induction. The percentage of callus was recorded after 8 weeks in culture. Physical factors such as light and dark treatments were employed to develop different stages of somatic embryos *in vitro*. Leaf and petiole explants were incubated in the dark for 8 weeks. To promote regeneration, embryogenic callus were transferred to either MS basal medium or hormone free or supplemented with 0.5 mg L^{-1} gibberelic acid (GA_3) and 0.2% activated charcoal. After somatic embryos had developed to cotyledonary stage, the clusters of somatic embryos were transferred to maturation medium MS containing 0.5 mg L^{-1} GA_3 and charcoal. After 8 weeks, the plantlets were acclimatized in the greenhouse for hardening process.

The experiment was conducted for 20 weeks before acclimatization take place and the cultures were incubated under 16 h photoperiod at 25±1°C. All treatments consisted of 12 replicates and each replicate contained two explants. For comparison of response of the two explants (leaf and petiole) of *B.* × *hiemalis* Fotsch. var. *Schwabenland Red* to different combinations of BAP and 2, 4-D, different concentration of casein hydrolysate and the influence of light and dark treatment, statistical analysis were done. The data was subjected to ANOVA test and each treatment mean was compared by critical difference at 0.05% level of significance.

RESULTS AND DISCUSSION

Based on preliminary studies it was found that 2, 4-D induced only non-embryogenic callus and did not develop into embryo. From the present work, the results showed that combinations of BAP and 2, 4-D was found to be the best for somatic embryogenesis induction in Begonia. Leaf and petiole explants produced callus *in vitro* on MS media supplemented with combinations of BAP and 2, 4-D. The colour of the callus formed was more dependent on different concentrations of BAP and 2, 4-D than on the different types of explants used. Most of the callus was green, yellowish, compact and nodular in structure. The explants enlarged and callus tissues were initiated from the cut end of the petiole and leaf explants. The initiation of callus started 1-2 weeks after inoculation and 4-6 weeks after culture establishment, callus subsequently covered the entire surfaces of the explants. The cultures were maintained at 25±1°C with 16 h light and 8 h dark and were subcultured every 6 weeks.

The percentage of callus obtained from leaf and petiole explants were determined. The results revealed that different types of explants produced different amount of callus. The leaf explants produced significantly more callus than petiole explants. Mean percentage of callus was not significantly different when different concentrations of BAP and 2, 4-D were used (Table 1).

Table 1: Mean percentage of callus obtained from leaf and petiole explants of *Begonia* × *hiemalis* Fotsch. with different concentrations of BAP and 2,4-D

Combination of BAP and 2,4-D (mg L^{-1})		Mean percentage of callus (%)			
BAP	2,4-D	Leaf explant	Callus color	Petiole explant	Callus color
0.1	0.1	55.83±3.12^{d}	Yellowish, green	44.16±1.49^{b}	Yellowish, green
0.5		57.50±3.05^{d}		25.00±1.95^{d}	
1.0		77.50±2.50^{b}		25.00±1.51^{d}	
1.5		28.33±1.12^{e}		15.00±1.51^{e}	
2.0		23.33±1.42^{e}		7.92±0.74^{f}	
0.1	0.5	80.00±2.13^{b}	Yellowish, green	27.50±3.29^{d}	Yellowish
0.5		91.25±2.23^{a}		61.67±6.49^{a}	
1.0		80.00±0.00^{b}		38.33±6.13^{b}	
1.5		70.00±2.13^{c}		33.33±3.96^{c}	
2.0		91.25±2.62^{a}		13.33±1.42^{e}	
0.1	1.0	87.50±4.46^{a}	Yellowish	47.92±6.26^{b}	Yellowish
0.5		87.50±4.27^{a}		35.83±5.83^{c}	
1.0		65.00±7.33^{c}		45.00±3.37^{b}	
1.5		52.50±3.51^{d}		25.00±5.11^{d}	
2.0		80.00±5.77^{b}		24.58±4.32^{d}	
0.1	1.5	80.00±4.44^{b}	Yellowish	15.83±1.93^{e}	Yellowish
0.5		70.00±4.61^{c}		12.50±1.69^{e}	
1.0		80.00±4.92^{b}		7.92±1.30^{f}	
1.5		55.00±4.69^{d}		8.33±2.33^{f}	
2.0		79.16±5.70^{b}		5.42±0.42^{f}	
0.1	2.0	85.83±2.28^{a}	Yellowish	7.08±1.30^{f}	Yellowish
0.5		48.33±7.47^{d}		7.50±1.69^{f}	
1.0		69.16±6.33^{c}		5.83±0.56^{f}	
1.5		83.75±6.72^{b}		37.50±2.50^{f}	
2.0		71.66±5.48^{c}		7.50±1.69^{f}	

*Values followed by the same letter(s) in the columns are not significantly different at $p<0.05$

Fig. 1: A-E: Somatic embryos produced from leaf explants of Begonia x *hiemalis* Fotsch. var. *Schwabenland Red* cultured on MS medium supplemented with 1.0 mg L^{-1} BAP and 0.1 mg L^{-1} 2,4- D, 500 mg L^{-1} casein hydrolysate and incubated in the dark for 8 weeks. (A) Globular somatic embryos developed from leaf explants after 5-6 weeks on induction medium, (B) Different stages of somatic embryos after 2-3 weeks on development medium (G-globular, H-heart-shape, T-torpedo and C-cotyledonary stage), (C) SEM of different stages of somatic embryos, (D) Cotyledonary stage of somatic embryos cultured into maturation medium, (E) *In vitro* plantlets derived from somatic embryos and (F) Normal plant derived from somatic embryogenesis process was successfully acclimatized in the greenhouse

Table 2: Mean percentage of embryogenic callus from leaf and petiole explants of *Begonia* × *hiemalis* Fotsch. var. *Schwabenland Red* cultured on MS medium supplemented with 1.0 mg L^{-1} BAP and 0.1 mg L^{-1} 2,4-D with different concentrations of casein hydrolysate. Data were obtained after 8 weeks of inoculation

Casein hydrolysate (mg L^{-1})	Callus induction % (±SE)		Embryogenic callus % (±SE)	
	Leaf	Petiole	Leaf	Petiole
Control	45.00±2.30^a	49.17±3.58^a	10.44±1.79^b	25.00±2.61^a
100	53.08±4.87^a	40.00±0.00^a	24.16±3.79^a	20.00±0.00^a
200	40.00±4.60^a	23.33±1.42^b	8.33±3.66^c	10.83±0.56^b
300	28.33±2.71^b	25.83±1.49^b	1.67±1.12^c	11.67±0.71^b
400	29.16±3.53^b	29.17±0.83^b	16.67±3.96^b	11.25±0.65^b
500	30.83±2.88^b	25.00±1.50^b	23.75±3.20^a	12.27±0.79^b

*Values followed by the same superscript letter(s) in the columns are not significantly different at $p<0.05$

Table 3: The effect of different light treatment on the production of embryogenic callus from leaf and petiole explants of *Begonia* × *hiemalis* Fotsch. var. *Schwabenland Red* cultured on MS medium supplemented with 1.0 mg L^{-1} BAP, 0.1 mg L^{-1} 2,4-D and 0.5 g L^{-1} casein hydrolysate. Data were obtained after 8 weeks of inoculation

Light treatment	Observations		Embryogenic callus % (±SE)	
	Leaf	Petiole	Leaf	Petiole
16 h light 8 h dark	Green nodular callus	Green nodular callus	58.67±4.68^a	36.00±1.31^a
24 h dark	White nodular callus	White nodular callus	40.44±4.68^a	40.67±5.30^a

Values followed by the same superscript letter(s) in the columns are not significantly different at $p<0.05$

The best induction of direct somatic embryogenesis of *Begonia* × *hiemalis* Fotsch.var. *Schwabenland Red* was achieved on MS medium supplemented with 1.0 mg L^{-1} BAP, 0.1-0.5 mg L^{-1} 2,4-D, 3% sucrose and solidified with 0.2% phytagel. However, Castillo and Smith (1977) reported that 0.5 mg L^{-1} kinetin and 2% coconut water were effective in inducing direct somatic embryogenesis in *B.* × *gracilis* explants.

Addition of casein hydrolysate in the callus induction medium was found to be beneficial and different concentrations of casein hydrolysate were also identified to optimize somatic embryo induction. The mean percentage of callus formation was presented in Table 2. Several reports also have proved the use of casein hydrolysate as beneficial for the formation of somatic embryos *in vitro* (Augustine and D'Souza, 1997; Ling *et al*., 1983; Narayanaswamy, 1997).

The effects of different light treatment on the production of somatic embryogenesis were also investigated. The results showed that mean percentage of callus was not significantly different from different explants for different light treatment (Table 3). Dark incubation produced complete embryogenesis cycle compared with 16 h light 8 h dark incubation (Fig. 1A-D). Augustine and D'Souza (1997) also reported that callus incubation in the dark could give rise to a large number of immature embryos.

The embryogenic callus was successfully regenerated after being transferred to MS medium supplemented with 0.5 mg L^{-1} GA_3 and 0.2% activated charcoal. For development of somatic embryos into maturation, withdrawal of BAP and 2, 4-D from induction medium was necessary. The withdrawal of BAP and 2,4-D resulted in the growth of embryos into plantlets. In conclusion, the present research succeeded in inducing somatic embryos from leaf and petiole explants of Begonia and subsequently regeneration of embryogenic callus.

CONCLUSION

The formation of somatic embryo in *Begonia* × *hiemalis* Fotsch. var. *Schwabenland Red* was successfully induced in MS medium supplemented with 1.0 mg L^{-1} BAP, 0.1-0.5 mg L^{-1} 2,4-D, 500 mg L^{-1} casein hydrolysate, 3% sucrose, solidified with 0.2% phytagel and incubated in the dark. Subsequently, different stages of somatic embryo development were initiated to form globular, heart-shape, torpedo and cotyledonary stage before forming *in vitro* plantlets.

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Growth Optimization and Organogenesis of *Gerbera jamesonii* Bolus ex. Hook f. *in vitro*

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Abstract: Regeneration potentials in *Gerbera jamesonii* Bolus ex. Hook f. from tissues culture system was studied using leaf, petiole and root explants. *In vitro* regeneration, callus induction and root formation were optimized by manipulation of growth regulators during organogenesis. Various kinds of plant growth regulators such as 6-Benzylaminopurine (BAP), α-Naphthalene acetic acid (NAA), 2, 4-Dichlorophenoxyacetic acid (2,4-D), Indole-3-acetic acid (IAA), Indole-3-Butyric acid (IBA), N^6-[2-Isopentenyl]adenine (2iP), Kinetin and Zeatin were used to initiate cultures. These plant growth regulators were added to Murashige and Skoog medium in different combinations and concentrations. Adventitious shoots were obtained from petiole explants cultured on Murashige and Skoog (MS) medium supplemented with 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA. Effectiveness of shoot regeneration medium, type of growth regulator used and duration of induction period were investigated. Leaf explants cultured on MS medium supplemented with 1.0 mg L^{-1} BAP and 2.0 mg L^{-1} 2, 4-D showed the best results for callus induction. Root explants were found to be non-regenerative in all experiments conducted. Petiole segment was identified as the best explant for regeneration of this species. Regenerated plants were rooted on Murashige and Skoog basal medium. Plantlets were then transferred to field with 75% survival rate.

Key words: *Gerbera jamesonii*, organogenesis, *in vitro*, callus induction, Murashige and Skoog

INTRODUCTION

Tissue culture technique has long been used since the cell theory was established. Many scientists have tried to prove the totipotency concept, which is the ability of a single cell to form complete individual. Today, tissue culture technique is being used widely realizing its potentials in mass propagation and preservation of elite plants. The most important aspect in plant tissue culture is the capability of cultured cells and tissues to regenerate into complete plants. This technology is being utilized commercially in the ornamentals industry and in other plant production organizations worldwide (Chu, 1992; Huetteman and Preece, 1993; Mantell *et al*., 1985; Pierik, 1987). Many temperate and tropical plants have been successfully propagated via tissue culture. *In vitro* response of plant tissues depends on genotype, the physiological status of the donor plant, the type of explant, the culture medium and their interactions (Tosca *et al*., 1999). The physiological status of the donor is determined by environmental conditions such as temperature, light intensity, day-length and light wavelength. Plant regeneration via direct organogenesis is much preferred over regeneration through somatic embryogenesis and callus culture (Arockiasamy *et al*., 2002). Adventitious organogenesis or shoot formation is a preferred system as it enables to retain the clonal fidelity since many ornamental species are cultivars that are propagated for one or more unique characteristics (Kantia and Kothari, 2002). The propagation rates via organogenesis can be much higher than axillary shoot proliferation (Chun, 1993).

In the present study, propagation of *Gerbera jamesonii* through tissue culture techniques was done and the factors influencing the growth of this plant were studied. *Gerbera jamesonii* Bolus ex. Hook f., commonly known as *Gerbera* daisy or Barberton daisy is a temperate perennial flowering plant which belongs to the Asteraceae family. They are planted outdoors in full sun and useful as cut flowers, pot plant and also bedding plant. This species consists of many cultivars with variety of colors and shapes and they are popular commercial plants. *Gerbera jamesonii* is also used in the preparation of traditional Chinese medicine, tu-er-feng, for curing cold and also for treating rheumatism (Ye *et al*., 1990). Plant propagation by tissue culture technique is mainly aimed to produce plants with very high multiplication rates. Through indirect organogenesis, multiple shoots can be produced *in vitro* from callus. Because genetic variability within the *Gerbera* genus is relatively limited, breeding

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potential for new flower colours and patterns as well as resistance to biotic or abiotic stresses is also limited (Orlikowska *et al*., 1999).

In the present study, experiments were conducted to investigate organogenesis from various sources of *Gerbera* explants. The effects of various concentrations of plant growth regulators on the multiplication of shoots were examined.

MATERIALS AND METHODS

The study was conducted at laboratory B2.5 and the green house at the Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia. Explants were obtained from 8-week-old aseptic seedlings. *Gerbera* seeds were first soaked in distilled water for 30 min with addition of 1-2 drops of Tween-20 followed by 40% (v/v) sodium chloride solution and gently agitated. The seeds were then rinsed 3 times with distilled water and then soaked in 70% (v/v) alcohol for 1 min. Finally the seeds were rinsed 3 times with sterile distilled water. Sterilized seeds were cultured on basal MS (Murashige and Skoog, 1962) medium. pH of the medium was adjusted at 5.8 before being autoclaved at 121°C for 21 min. Petioles and leaves obtained from aseptic young plantlets formed from the seedlings were used as source of explants. Leaves and petioles were cultured for shoot induction on MS medium supplemented with 3% sucrose and 0.8% technical agar containing 6-Benzylaminopurine, BAP (0.5-2.0 mg L^{-1}) and α-Naphthalene acetic acid, NAA (0.5-1.0 mg L^{-1}). Induction of rooting *in vitro* was also observed when petiole and leaf explants were cultured on MS medium fortified with BAP, 2, 4-D, NAA, Zeatin, IAA and 2iP (Fig. 1). Callus induction was initiated on MS medium with BAP, Kinetin, Zeatin and 2iP (0.5-2.0 mg L^{-1}) and NAA, IAA, IBA and 2, 4-D (0.5-2.0 mg L^{-1}).

Plantlets were transferred to soil and maintained in culture room for 2-3 weeks for adaptation process before being transferred to field environment. *In vitro* propagated *Gerbera* plants were compared morphologically with the intact plant. Survival rate of micro propagated plants that were transferred to soil were investigated (Fig. 2).

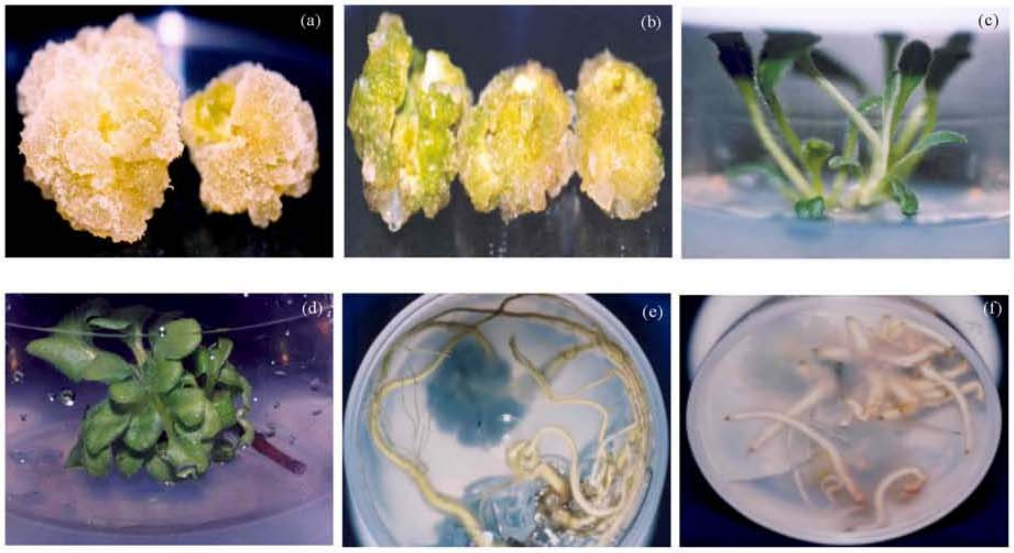

Fig. 1: (a) Callus formed on MS medium supplemented with 1.0 mg L^{-1} BAP and 2.0 mg L^{-1} 2,4-D, (b) Callus formed on MS medium added with 1.0 mg L^{-1} BAP and 2.0 mg L^{-1} NAA, (c) Regeneration of shoots from petiole explants cultured on MS medium supplemented with 2.0 mg L^{-1} Zeatin and 0.5 mg L^{-1} IBA, (d) Regeneration of shoots from petiole explants cultured on MS medium supplemented with 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA, (e) Development of roots from leaf explant cultured on MS medium supplemented with 0.1 mg L^{-1} BAP and 2.0 mg L^{-1} NAA and (f) Roots formed from leaf explants cultured on MS medium fortified with 2.0 mg L^{-1} NAA

Fig. 2: (a) Plantlet ready to be transferred to soil, (b) Young *Gerbera* plantlets were transferred to soil, (c) *Gerbera* plantlet after 8 weeks being transferred to soil and (d) Six- months-old *Gerbera* plant successfully acclimatized

RESULTS AND DISCUSSION

Plant hormones and type of explants play very important roles in determining regeneration of *Gerbera jamesonii in vitro*. Many commercial ornamental plants are being propagated by *in vitro* culture on the culture medium containing auxins and cytokinins (Preil, 2003; Rout and Jain, 2004). Ornamental plants and woody plant species are also propagated commercially by axillary bud proliferation (Mantell *et al*., 1985; Pierik, 1987; Chu, 1992). Various explants have been used for direct shoot formation. Different types of auxin and cytokinin combinations were used in order to obtain complete regeneration of *Gerbera in vitro*. BAP strongly enhanced regeneration of shoots in petiole explants of *Gerbera jamesonii*. The right combination of auxin and cytokinin in the culture medium determined the effectiveness of micro propagation of *Gerbera* shoots. In the present study, regeneration of shoots increased when BAP was added with NAA. Highest numbers of shoots from petiole explants (9.3±0.6 per explant) were obtained when explants were cultured on MS medium supplemented with 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA (Table 1). Shoots were obtained after 28 days of culture and shoot growth was normal. The lowest shoot formation was observed when explants were cultured on MS medium supplemented with 0.1 mg L^{-1} BAP and 2.0 mg L^{-1} NAA with 1.6±0.7 shoots per explant (Table 1).

Table 1: Percentage of shoot formation and number of shoots per explants produced on MS medium supplemented with different hormones and concentrations after 8 weeks in culture

MS+hormone (mg L^{-1})	Explants	Shoot regeneration (%)	No. of shoots per explants
2.0 mg L^{-1} BAP+0.5 mg L^{-1} NAA	Petiole	94.3±2.5	9.3±0.6
2.0 mg L^{-1} BAP+1.5 NAA	Petiole	79.2±2.0	5.2±1.2
2.0 mg L^{-1} Zeatin+0.5 mg L^{-1} IBA	Petiole	83.7±1.5	7.4±0.9
0.1 mg L^{-1} BAP+2.0 mg L^{-1} NAA	Petiole	4.6±0.8	1.6±0.7
1.0 mg L^{-1} BAP+2.0 mg L^{-1} NAA	Petiole	15.4±2.7	3.5±1.8
1.5 mg L^{-1} BAP+1.0 mg L^{-1} NAA	Petiole	83.1±0.5	8.3±1.1
0.5 mg L^{-1} BAP+0.5 mg L^{-1} NAA	Petiole	33.4±1.2	4.7±0.5
2.0 mg L^{-1} BAP+0.5 mg L^{-1} IAA	Petiole	33.6±3.4	3.5±2.6
2.0 mg L^{-1} BAP+0.5 mg L^{-1} IBA	Petiole	39.7±2.1	5.1±1.3
2.0 mg L^{-1} Kinetin+0.5 mg L^{-1} IBA	Petiole	66.7±0.8	6.2±0.7
2.0 mg L^{-1} Zeatin+0.5 mg L^{-1} 2,4-D	Petiole	22.1±1.5	2.4±1.6
2.0 mg L^{-1} Zeatin+0.5 mg L^{-1} IAA	Petiole	73.2±1.4	5.6±0.8
2.0 mg L^{-1} 2ip+0.5 mg L^{-1} IBA	Petiole	11.9±2.3	2.9±1.7
2.0 mg L^{-1} 2ip+0.5 mg L^{-1} IAA	Petiole	26.3±2.0	2.4±1.0
3.0 mg L^{-1} BAP	Petiole	80.4±1.6	8.8±0.9

Addition of strong auxin (NAA) with BAP promoted better shoot formation compared to weak auxin (IAA) (Pierik *et al*., 1973). However, higher addition of auxin compared to cytokinin in the culture medium resulted in the inhibition of shoot formation. Shoot formation was observed when capitulum explants were cultured on MS modified medium supplemented with 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} IAA (Eduardo *et al*., 1991). Jones *et al*. (2007) reported that the supplementation of TDZ (thidiazuron) in culture medium resulted shoots regeneration in *Echinacea purpurea* L. Lower concentration of TDZ promoted direct shoot regeneraration and higher concentrations promoted callus induction in *Hagenia abyssinica* (Bruce) J.F. Gmel. (Feyissa *et al*., 2005). The present study showed that only petiole explant produced optimum results for shoot formation. Capitulum explant promoted callus development. Vardja and Vardja (2001) reported that, the addition of 10 mg L^{-1} BAP in culture medium increased the formation of adventitious buds of *Gerbera* at initiation and multiplication stage. However, in this study, at a very high concentration of BAP and other growth regulators, development and growth of shoots were inhibited.

It is reported that a good combination of cytokinin and auxin in the culture medium enhanced good shoot formation and plantlet regeneration from explants, for example, adventitious *Gerbera* shoots were regenerated from flower buds of greenhouse grown plants (Pierik *et al*., 1973, 1975; Laliberte *et al*., 1985). Martin *et al*. (2003) obtained indirect shoot bud regeneration from lamina explants of *Anthurium andraeanum* on MS medium supplemented with 0.25 mg L^{-1} BAP, 0.2 mg L^{-1} IAA and 0.1 mg L^{-1} Kinetin. Takayama and Misawa (1982) reported that medium containing 0.3 mg L^{-1} BAP or 0.1 mg L^{-1} Kinetin along with 1.0 mg L^{-1} NAA showed rapid regeneration of shoot buds from leaf and petiole segments of *Begonia* sp. The combination of low concentration of cytokinin and auxin initiated propagation of *Begonia* species (Reuter and Bhandari, 1981). Cytokinin alone in the culture medium induces shoot formation in many plants. However, in *Gerbera jamesonii*, cytokinin alone in the medium induces the formation of microshoots. Combination of auxin and cytokinin induces the formation of adventitious shoots and roots. Addition of auxins together with cytokinins becomes essential for shoot induction and multiplication depending on the plant type. Micropropagation of *Saintpaulia ionantha* was optimized when petiole explants were cultured on MS medium supplemented with 2.0 mg L^{-1} Zeatin and 1.0 mg L^{-1} IAA (Daud, 2005). Rancillac and Nourrisseau (1989) improved the performance of micropropagated strawberry plants by decreasing the cytokinin concentration and limiting the number of subcultures. High concentrations of cytokinin in culture medium were found to be unsuitable for shoot formation from leaf or petiole explants in some ornamentals. *Gerbera* plantlets formed were transferred to soil with 75.03% survival rate. The regenerated plants were observed to be normal.

The first step towards de novo regeneration is to establish callus or cell suspension cultures. Tissues of explants generally show distinct planes of cell division, various specializations of cells and organization into specialized structures such as the vascular system. Formation of callus from explants tissues involves the development of progressively more random planes of cell division, less frequent specialization of cells and loss of organized structures (Thorpe, 1980; Wagley *et al*., 1987). In *Gerbera jamesonii*, BAP was also required for the formation of callus. Based on the present study, all combinations of hormones used were able to induce callus. Callus with good and compact structure was obtained when explant was cultured on MS medium supplemented with the combination of BAP and 2, 4-D. Relatively, in this experiment, callus formation was optimum when leaf explant was cultured on MS medium supplemented with 1.0 mg L^{-1} BAP+2.0 mg L^{-1} 2, 4-D (Table 2). This callus gave the highest dry weight. Callus

Table 2: Percentage of callus and root formation per explants produced on MS medium supplemented with different hormones and concentration after 8 weeks in culture

MS+hormone (mg L^{-1})	Explants	Callus (%)	Root (%)	No. of roots per explant
2.0 mg L^{-1} BAP+	Leaf	89.4± 2.3	0	0
0.5 mg L^{-1} NAA	Root	67.5± 4.7	0	0
2.0 mg L^{-1} Zeatin+	Leaf	65.8±3.3	2.5±0.7	0.8±0.7
0.5 mg L^{-1} IBA	Root	31.7±5.2	0	0
0.1 mg L^{-1} BAP+	Leaf	58.9±3.1	73.7±2.3	22.1±2.3
2.0 mg L^{-1} NAA	Root	49.7±3.9	63.5±0.5	19.1±0.5
0.5 mg L^{-1} BAP+	Leaf	86.3±0.6	71.3±1.5	21.4±1.5
2.0 mg L^{-1} NAA	Root	58.3±1.6	65.0±0.9	19.5±0.9
1.0 mg L^{-1} BAP+	Leaf	90.7±0.9	21.4±2.1	6.4±2.1
2.0 mg L^{-1} NAA	Root	79.3±3.4	26.3±1.8	7.9±1.8
1.5 mg L^{-1} BAP+	Leaf	87.3±0.6	1.2±0.8	0.4±0.8
1.0 mg L^{-1} NAA	Root	55.6±1.2	7.4±0.5	2.2±0.5
2.0 mg L^{-1} BAP+	Leaf	66.3±2.4	0	0
0.5 mg L^{-1} IAA	Root	42.8±3.6	0	0
2.0 mg L^{-1} BAP+	Leaf	78.4±2.7	0	0
0.5 mg L^{-1} IBA	Root	29.1±3.2	0	0
1.0 mg L^{-1} BAP+	Leaf	98.4±0.6	0	0
2.0 mg L^{-1} 2,4-D	Root	83.2±1.5	0	0
2.0 mg L^{-1} BAP+	Leaf	87.1±2.2	0	0
2.0 mg L^{-1} 2,4-D	Root	60.4±1.8	0	0
1.0 mg L^{-1} BAP+	Leaf	57.0±2.0	0	0
0.1 mg L^{-1} 2,4-D	Root	30.5±1.0	0	0
2.0 mg L^{-1} IAA+	Leaf	63.6±2.3	54.7±1.7	16.4±1.7
0.5 mg L^{-1} 2iP	Root	43.5±1.9	23.1±2.1	7.0±2.1
2.0 mg L^{-1} IBA+	Leaf	86.5±1.2	16.7±1.6	5.0±1.6
2.0 mg L^{-1} 2iP	Root	50.3±2.4	9.6±1.0	2.9±1.0
2.0 mg L^{-1} 2,4-D+	Leaf	91.3±1.6	68.4±0.5	20.5±0.5
0.5 mg L^{-1} Zeatin	Root	66.4±2.3	0	0
3.0 mg L^{-1} BAP	Leaf	5.3±0.5	0	0
	Root	2.6±1.7	0	0
2.0 mg L^{-1} NAA	Leaf	10.4±2.4	70.7±2.4	21.2±2.4
	Root	5.5±0.3	60.5±1.0	18.1±1.0

formed in these experiments were mainly green. However, white, creamy friable callus was obtained when explants were cultured on MS medium supplemented with 2, 4-D alone. The lowest callus formation was obtained when root explant was cultured on MS medium supplemented with 3.0 mg L^{-1} BAP (Table 2). In *Begonia*, the addition of kinetin and zeatin in the culture medium (Murashige and Skoog, 1962) induced multiple shoots (Jain, 1997). Callus is capable of forming adventitious roots. Root formation occurred when explants were cultured on medium with higher auxin concentration and lower cytokinin concentration. Highest root formation was obtained when leaf explants were cultured on MS medium supplemented with 0.1 mg L^{-1} BAP and 2.0 mg L^{-1} NAA with an average of 22.1±2.3 roots per explants (Table 2). However, regeneration of root was not observed when explants were cultured on MS medium supplemented with BAP and 2, 4-D. Bigot (1981) reported that the addition of 1-2 g L^{-1} activated charcoal in the culture medium showed vigorous rooting from excised shoots of *Begonia*× *hiemalis*. However, in *Gerbera*, addition of charcoal is not necessary for rooting.

Roest *et al*. (1981) successfully induced roots in the adventitious shoots of chrysanthemum in the liquid MS medium containing 1.0 mg L^{-1} IAA. Shoots and roots of chrysanthemum were developed on a single medium containing 1.0 mg L^{-1} BAP and 1.2 mg L^{-1} kinetin. In this study, roots were induced from adventitious shoots of *Gerbera jamesonii* on solid MS medium fortified with various concentration and combination of BAP, NAA, IBA, 2iP and Zeatin. Root explants itself also induced the formation of root *in vitro*.

Rooted plantlets of *Gerbera* were successfully established and all plantlets were transferred to soil and maintained in the green house. Plantlets survival rate achieved was 75.03%.

CONCLUSIONS

The main purpose for propagation of ornamental plants is for its aesthetic value. Thus, improvements of plant quality need to be studied and more research needs to be done. One of the most important techniques in plant improvements is via micro propagation. Successful *in vitro* propagation of ornamental plants is now being widely used in commercialization purposes. In conclusion, the research done has proven that micro propagation of *Gerbera jamesonii* Bolus ex. Hook f. *in vitro* could be successfully obtained. Petiole explants have been identified as the most regenerative explants for multiple shoot formation. Studies of *Gerbera* clonal propagation could also be efficiently adapted for other ornamental plants.

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